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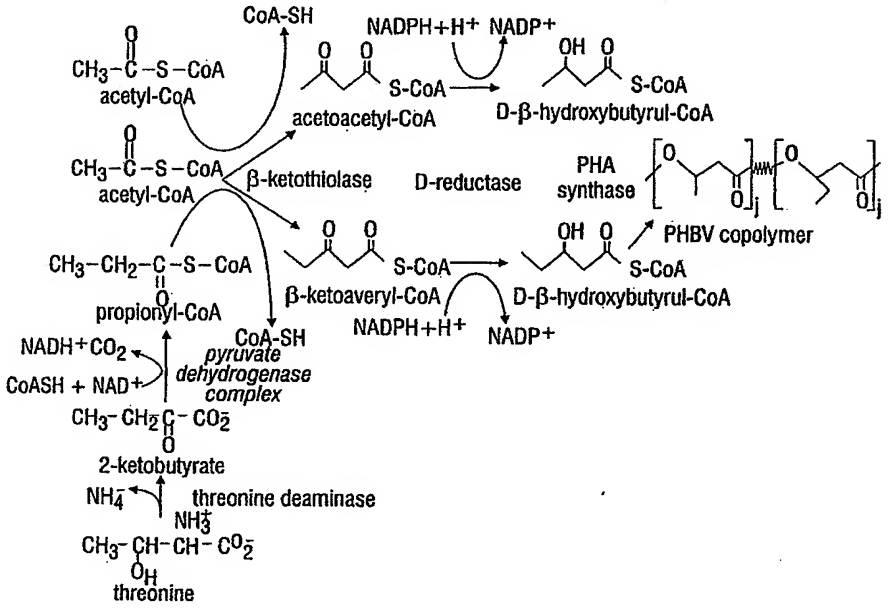

(57) Abstract

The use of multigene vectors for the preparation of transformed host cells and plants is disclosed. Multigene vectors reduce the number of transformations required, and leads to increased production of polyhydroxyalkanoate polymer in the resulting transformed host cells and plants.

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MULTIGENE EXPRESSION VECTORS FOR THE BIOSYNTHESIS OF PRODUCTS VIA MULTIENZYME BIOLOGICAL PATHWAYS

This application is based on United States Provisional Application No. 60/123,015, filed March 5, 1999.

FIELD OF THE INVENTION

The invention relates to the construction and use of multigene expression vectors useful to enhance production of materials by multienzyme pathways. In particular, the construction and use of multigene vectors encoding proteins in the polyhydroxyalkanoate biosynthetic pathway is disclosed.

BACKGROUND OF THE INVENTION

Metabolic engineering is a process by which the normal metabolism of an organism is altered to change the concentration of normal metabolites, or to create novel metabolites. This process often involves introduction or alteration of numerous enzymatic steps, and thus often requires introduction of multiple genes. An efficient system for introducing and expressing multiple genes is therefore desirable. In prokaryotes such as *Escherichia coli*, introduction of multiple genes is relatively straightforward in that operons can be constructed to express multiple open reading frames, or multiple complete genes can be expressed from a single plasmid. However, introduction of pathways into plants is more difficult due in part to the complexity of plant genes, the difficulty of constructing vectors harboring multiple genes for expression in plants, and the difficulty of introducing large vectors intact into plants.

Polyhydroxyalkanoates are bacterial polyesters that accumulate in a wide variety of bacteria. These polymers have properties ranging from stiff and brittle plastics to rubber-like materials, and are biodegradable. Because of these properties, polyhydroxyalkanoates are an attractive source of non-polluting plastics and elastomers.

Currently, there are approximately a dozen biodegradable plastics in commercial use that possess properties suitable for producing a number of specialty and commodity products (Lindsay, *Modern Plastics* 2: 62, 1992). One such biodegradable plastic in the

polyhydroxyalkanoate (PHA) family that is commercially important is Biopol™, a random copolymer of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV). This bioplastic is used to produce biodegradable molded material (e.g., bottles), films, coatings, and in drug release applications. Biopol™ is produced via a fermentation process employing the bacterium *Ralstonia eutropha* (Byrom, D. *Trends Biotechnol.* 5: 246-250, 1987). (*R. eutropha* was formerly designated *Alcaligenes eutrophus* [Yabuuchi et al., *Microbiol. Immunol.* 39:897-904, 1995]). The current market price is $6-7/lb, and the annual production is 1,000 tons. By best estimates, this price can be reduced only about 2-fold via fermentation (Poirier, Y. et al., *Bio/Technology* 13: 142, 1995). Competitive synthetic plastics such as polypropylene and polyethylene cost about 35-45¢/lb (Layman, *Chem. & Eng. News*, p. 10 (Oct. 31, 1994). The annual global demand for polyethylene alone is about 37 million metric tons (Poirier, Y. et al., *Int. J. Biol. Macromol.* 17: 7-12, 1995). It is therefore likely that the cost of producing P(3HB-co-3HV) by microbial fermentation will restrict its use to low-volume specialty applications.

Polyhydroxyalkanoate (PHA) is a family of polymers composed primarily of R-3-hydroxyalkanoic acids (Anderson, A. J. and Dawes, E. A. *Microbiol. Rev.* 54: 450-472, 1990; Steinbüchel, A. in *Novel Biomaterials from Biological Sources*, ed. Byrom, D. (MacMillan, New York), pp. 123-213, 1991); Poirier, Y., Nawrath, C. & Somerville, C. *Bio/Technology* 13: 143-150, 1995). Polyhydroxybutyrate (PHB) is the most well-characterized PHA. High molecular weight PHB is found as intracellular inclusions in a wide variety of bacteria (Steinbüchel, A. in *Novel Biomaterials from Biological Sources*, ed. Byrom, D. (MacMillan, New York), pp. 123-213, 1991). In *Ralstonia eutropha*, PHB typically accumulates to 80% dry weight with inclusions being typically 0.2-1 μm in diameter. Small quantity of PHB oligomers of approximately 150 monomer units are also found associated with membranes of bacteria and eukaryotes, where they form channels permeable to calcium (Reusch, R. N., *Can. J. Microbiol.* 41 (Suppl. 1): 50-54, 1995). High molecular weight polyhydroxyalkanoates have the properties of thermoplastics and elastomers. Numerous bacteria and fungi can hydrolyze polyhydroxyalkanoates to monomers and oligomers, which are metabolized as a carbon source. Polyhydroxyalkanoates have accordingly attracted attention as a potential source of renewable and biodegradable plastics and elastomers. PHB is a highly crystalline polymer with rather poor physical properties, being relatively stiff and brittle (de Koning, G., *Can. J. Microbiol.* 41 (Suppl. 1): 303-309, 1995). In contrast, PHA copolymers containing monomer units ranging

from 3 to 5 carbons for short-chain-length PHA (SCL-PHA), or 6 to 14 carbons for medium-chain-length PHA (MCL-PHA), are less crystalline and more flexible polymers (de Koning, G., *Can. J. Microbiol.* 41 (Suppl. 1): 303-309, 1995).

PHB has been produced in the plant *Arabidopsis thaliana* expressing the *R. eutropha* PHB biosynthetic enzymes (Poirier, Y. et al., *Science* 256: 520-523, 1992; Nawrath, C., et al., *Proc. Natl. Acad. Sci. U.S.A.* 91: 12760-12764, 1994). In plants expressing the PHB pathway in the plastids, leaves accumulated up to 14% PHB per gram dry weight (Nawrath, C., et al., *Proc. Natl. Acad. Sci. U.S.A.* 91: 12760-12764, 1994). High-level synthesis of PHB in plants opened the possibility of utilizing agricultural crops as a suitable system for the production of polyhydroxyalkanoates on a large scale and at low cost (Poirier, Y. et al., *Bio/Technology* 13: 143-150, 1995; Poirier, Y. et al., *FEMS Microbiol. Rev.* 103: 237-246, 1992; Nawrath, C., et al. *Molecular Breeding* 1: 105-22, 1995). PHB was also shown to be synthesized in insect cells expressing a mutant fatty acid synthase (Williams, M. D., et al., *Appl. Environ. Microbiol.* 62: 2540-2546, 1996), and in yeast expressing the *R. eutropha* PHB synthase (Leaf, T. A., et al. *Microbiol.* 142: 1169-1180, 1996).

A number of pseudomonads, including *Pseudomonas putida* and *Pseudomonas aeruginosa*, accumulate MCL-PHAs when cells are grown on alkanoic acids (Anderson, A. J. & Dawes, E. A. *Microbiol. Rev.* 54: 450-472, 1990; Steinbüchel, A. in *Novel Biomaterials from Biological Sources*, ed. Byrom, D. (MacMillan, New York), pp. 123-213, 1991; Poirier, Y., Nawrath, C. & Somerville, C. *Bio/Technology* 13: 143-150, 1995). The nature of the PHA produced is related to the substrate used for growth and is typically composed of monomers which are 2n carbons shorter than the substrate. These studies indicate that MCL-PHAs are synthesized by the PHA synthase from 3-hydroxyacyl-CoA intermediates generated by the β-oxidation of alkanoic acids (Huijberts, G. N. M., et al. *Appl. Environ. Microbiol.* 58: 536-544, 1992; Huijberts, G. N. M., et al., *J. Bacteriol.* 176: 1661-1666, 1994).

Chen et al. (*Nature Biotech.*, 16: 1060-1064, 1998; reviewed by Gelvin, S.B., *Nature Biotech.*, 16: 1009-1010, 1998) describes the cobombardment of embryogenic rice tissues with a mixture of 14 different pUC based plasmids. Integration of multiple transgenes was observed to occur at one or two genetic loci.

Creating a transgenic host cell or plant that produces multiple enzymes within a biosynthetic pathway is often a daunting task. Individual vectors must be created for each

enzyme. Transformation of the host cell or plant is typically accomplished by one of three general methods: serial transformation, parallel transformation followed by crossing, or batch transformation. Each method has serious practical drawbacks.

Serial transformation involves transforming a host cell or plant with the first vector, selecting and characterizing the transformed cell or plant, transforming with the second vector, and so on. This process can become quite laborious and time consuming.

Parallel transformation followed by crossing involves separately transforming cells with each of the individual vectors, and subsequently mating or crossbreeding the transformed cells or plants to obtain a final cell or plant which contains all of the individual sequences. This is a lengthy process, especially for the crossbreeding of plant lines.

Batch transformation involves a single transformation event involving all of the individual vectors. A wide array of cells are produced, each containing between none and all of the vectors. While only a single transformation is required, extensive characterization of the resulting cells is necessary. As the number of vectors increases, it is increasingly likely that no cells will be obtained containing all of the vectors. If no desired transformed cells are identified, the transformation must be repeated.

An additional concern with all three of these methods is that they do not allow any control over the relative copy numbers of the individual vectors in the transformed cell or plant. It would be desirable to have a transformation method that permits control of the relative copy numbers of the individual sequences in the transformed cell or plant, and also coordinates the positional effect of the insertion locus.

There exists a need for improved materials and methods for the preparation of transgenic organisms transformed with multiple nucleic acid sequences encoding members of a multi-enzyme biosynthetic pathway.

SUMMARY OF THE INVENTION

The invention involves the construction and use of nucleic acid segments and vectors containing multiple sequences encoding members of a biosynthetic pathway. The resulting vector allows a single transformation event to produce a transformed cell or plant containing all of the nucleic acid sequences. Furthermore, the researcher has total control over the number of

copies of each coding sequence within the constructed vector. Single or multiple copies of each coding sequence may easily be designed into the vector.

An unexpected beneficial result of the invention is that organisms transformed with a multi-enzyme coding vector produce the biosynthetic product in higher yield than organisms produced by serial transformation, parallel transformation with crossing, or batch transformation methods.

DETAILED DESCRIPTION OF THE INVENTION

The invention is directed generally towards the construction and use of nucleic acid segments comprising sequences encoding multiple enzymes in a multi-enzyme biosynthetic pathway. The biosynthetic pathway may generally be any biosynthetic pathway. Examples of such multi-enzyme biosynthetic pathways are the TCA cycle, polyketide synthesis pathway, carotenoid synthesis, glycolysis, gluconeogenesis, starch synthesis, lignins and related compounds, production of small molecules that serve as pesticides, fungicides, or antibiotics, and polymer synthesis pathways. Preferably, the biosynthetic pathway is a polyhydroxyalkanoate biosynthesis pathway.

This disclosure describes multigene vectors designed to produce polyhydroxyalkanoate (PHA) in plants. Some of these vectors are designed to produce poly(β-hydroxybutyrate), and some are designed to produce poly(β-hydroxybutyrate-co-β-hydroxyvalerate) (Gruys et al., WO 98/00557, 1998). In general, the efficiency of PHA production was dramatically increased when all sequences necessary for a pathway were introduced on the same vector. Herein, construction of these multigene vectors, and their use for polyhydroxyalkanoate production in *Arabidopsis thaliana* and *Brassica napus*, and *Zea mays* is described.

An embodiment of the present invention is an isolated nucleic acid segment comprising multiple nucleic acid sequences, each encoding a different protein within the biosynthetic pathway. Preferably, the isolated nucleic acid segment comprises a first nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein; a second nucleic acid sequence encoding a β-ketoacyl reductase protein; and a third nucleic acid sequence encoding a β-ketothiolase protein. The nucleic acid segment may further comprise additional nucleic acid sequences

encoding additional proteins such as a threonine deaminase protein or a deregulated threonine deaminase protein.

An alternative embodiment of the invention is a recombinant vector comprising multiple nucleic acid sequences, each encoding a different protein within the biosynthetic pathway. The recombinant vector may be arranged with a single promoter producing a polycistronic RNA transcript from the multiple nucleic acid sequences, or with each nucleic acid sequence being under the control of its own promoter. The multiple promoters may be the same or different. It is also possible to have one or more nucleic acid sequence under the control of its own promoter, while other nucleic acid sequences may be jointly under the control of a single promoter producing a polycistronic RNA transcript.

A recombinant vector placing the biosynthetic pathway nucleic acid sequences under the control of a single promoter preferably comprises operatively linked in the 5' to 3' direction: a promoter that directs transcription of the first nucleic acid sequence, second nucleic acid sequence, and third nucleic acid sequence; a first nucleic acid sequence; a second nucleic acid sequence; a third nucleic acid sequence; a 3' transcription terminator; and a 3' polyadenylation signal sequence; wherein: the first nucleic acid sequence, second nucleic acid sequence, and third nucleic acid sequence encode different proteins; and the first nucleic acid sequence, second nucleic acid sequence, and third nucleic acid sequence are independently selected from the group consisting of a nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein, a nucleic acid sequence encoding a β-ketoacyl reductase protein, and a nucleic acid sequence encoding a β-ketothiolase protein. The nucleic acid sequences encoding the biosynthetic pathway enzymes may be in any order relative to each other and the promoter. The promoter must be expressed in plastids. It may have either been derived from a plastid, or may have been derived from a bacterium or phage having promoters recognized by the plastid transcription enzymes, or be a synthetic promoter recognized by the plastid transcription enzymes.

A recombinant vector placing the biosynthetic pathway nucleic acid sequences under the control of multiple promoters preferably comprises a first element comprising operatively linked in the 5' to 3' direction: a first promoter that directs transcription of the first nucleic acid sequence; a first nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein; a first 3' transcription terminator; a first 3' polyadenylation signal sequence; a second element comprising operatively linked in the 5' to 3' direction: a second promoter that directs

transcription of the second nucleic acid sequence; a second nucleic acid sequence encoding a β-ketoacyl reductase protein; a second 3' transcription terminator; a second 3' polyadenylation signal sequence; and a third element comprising operatively linked in the 5' to 3' direction: a third promoter that directs transcription of the third nucleic acid sequence; a third nucleic acid sequence encoding a β-ketothiolase protein; a third 3' transcription terminator; and a third 3' polyadenylation signal sequence. The β-ketothiolase protein preferably condenses two molecules of acetyl-CoA to produce acetoacetyl-CoA; and condenses acetyl-CoA and propionyl-CoA to produce β-ketovaleryl-CoA. The β-ketoacyl reductase protein preferably reduces acetoacetyl-CoA to β-hydroxybutyryl-CoA; and reduces β-ketovaleryl-CoA to β-hydroxyvaleryl-CoA. The polyhydroxyalkanoate synthase protein is preferably selected from the group consisting of: a polyhydroxyalkanoate synthase protein that incorporates β-hydroxybutyryl-CoA into P(3HB) polymer; and a polyhydroxyalkanoate synthase protein that incorporates a β-hydroxybutyryl-CoA and a β-hydroxyvaleryl-CoA into P(3HB-co-3HV) copolymer. The β-ketothiolase protein may comprise a transit peptide sequence that directs transport of the β-ketothiolase protein to the plastid. The β-ketoacyl reductase protein may comprise a transit peptide sequence that directs transport of the β-ketoacyl reductase protein to the plastid. The polyhydroxyalkanoate synthase protein may comprise a transit peptide sequence that directs transport of the polyhydroxyalkanoate synthase protein to the plastid. The recombinant vector may further comprise a nucleic acid sequence encoding a threonine deaminase protein or a deregulated threonine deaminase protein. The first promoter, second promoter, and third promoter are preferably active in plants. The first promoter, second promoter, and third promoter are preferably viral promoters. The first promoter, second promoter, and third promoter are preferably independently selected from the group consisting of a CMV 35S promoter, an enhanced CMV 35S promoter, maize chlorophyll A/B binding protein promoter, and an FMV 35S promoter. More preferably, the first promoter, second promoter, and third promoter are the CMV 35S promoter. The first promoter, second promoter, and third promoter may be tissue specific promoters. The first promoter, second promoter, and third promoter may independently be the *Lesquerella* hydroxylase promoter or the 7S conglycinin promoter, and preferably each is the *Lesquerella* hydroxylase promoter.

An alternative embodiment is directed towards transformed host cells. Transformed host cells may contain a non-integrated recombinant vector or an integrated recombinant vector.

A transformed host cell may comprise a recombinant vector, wherein the recombinant vector comprises a first element comprising operatively linked in the 5' to 3' direction: a first promoter that directs transcription of the first nucleic acid sequence; a first nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein; a first 3' transcription terminator; a first 3' polyadenylation signal sequence; a second element comprising operatively linked in the 5' to 3' direction: a second promoter that directs transcription of the second nucleic acid sequence; a second nucleic acid sequence encoding a β-ketoacyl reductase protein; a second 3' transcription terminator; a second 3' polyadenylation signal sequence; and a third element comprising operatively linked in the 5' to 3' direction a third promoter that directs transcription of the third nucleic acid sequence; a third nucleic acid sequence encoding a β-ketothiolase protein; a third 3' transcription terminator; and a third 3' polyadenylation signal sequence.

The transformed host cell may alternatively contain an integrated nucleic acid segment. Preferably, the transformed host cell may comprise a first element comprising operatively linked in the 5' to 3' direction: a first promoter that directs transcription of a first nucleic acid sequence; a first nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein; a first 3' transcription terminator; a first 3' polyadenylation signal sequence; a second element comprising operatively linked in the 5' to 3' direction: a second promoter that directs transcription of a second nucleic acid sequence; a second nucleic acid sequence encoding a β-ketoacyl reductase protein; a second 3' transcription terminator; a second 3' polyadenylation signal sequence; and a third element comprising operatively linked in the 5' to 3' direction: a third promoter that directs transcription of a third nucleic acid sequence; a third nucleic acid sequence encoding a β-ketothiolase protein; a third 3' transcription terminator; and a third 3' polyadenylation signal sequence. The first element, second element, and third element may be cointegrated within a continuous 10 Mb segment of genomic DNA, more preferably within a continuous 5 Mb, 2.5 Mb, 2 Mb, 1.5 Mb, 1 Mb, 500 kb, 250 kb, 100 kb, 50 kb, or 20 kb segment of genomic DNA. Alternatively, the first element, second element, and third element may be cointegrated between a left Ti border sequence and a right Ti border sequence. While it is preferable that a recombinant vector contain a single left Ti border sequence and a single right Ti border

sequence, the invention encompasses recombinant vectors containing multiple left and/or right Ti border sequences, and the use thereof.

Alternatively, the host cell may comprise a nucleic acid segment containing nucleic acid sequences encoding enzymes in a biosynthetic pathway, where a single promoter directs transcription of the nucleic acid sequences.

The transformed host cell may generally be any host cell, and preferably is a bacterial, fungal, or plant cell. The bacterial cell is preferably an *Escherichia coli* cell. The fungal cell is preferably a yeast, *Saccharomyces cerevisiae*, or *Schizosaccharomyces pombe* cell. The plant cell may be a monocot plant cell, a dicot plant cell, an algae cell, or a conifer plant cell. The plant cell is preferably a tobacco, wheat, potato, *Arabidopsis*, corn, soybean, canola, sugar beet, oil seed rape, sunflower, flax, peanut, sugarcane, switchgrass, or alfalfa cell.

The promoters may be any of the promoters discussed earlier. The transformed host cells preferably produce polyhydroxyalkanoate polymer.

The invention also encompasses transformed plants. The transformed plant may contain an integrated set of nucleic acid sequences, or may contain the same set of nucleic acid sequences on a non-integrated vector. A preferred embodiment is directed towards a transformed plant comprising a first element comprising operatively linked in the 5' to 3' direction: a first promoter that directs transcription of a first nucleic acid sequence; a first nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein; a first 3' transcription terminator; a first 3' polyadenylation signal sequence; a second element comprising operatively linked in the 5' to 3' direction: a second promoter that directs transcription of a second nucleic acid sequence; a second nucleic acid sequence encoding a β-ketoacyl reductase protein; a second 3' transcription terminator; a second 3' polyadenylation signal sequence; and a third element comprising operatively linked in the 5' to 3' direction: a third promoter that directs transcription of a third nucleic acid sequence; a third nucleic acid sequence encoding a β-ketothiolase protein; a third 3' transcription terminator; and a third 3' polyadenylation signal sequence. The first element, second element, and third element may be cointegrated within a continuous 10 Mb segment of genomic DNA, more preferably within a continuous 5 Mb, 2.5 Mb, 2 Mb, 1.5 Mb, 1 Mb, 500 kb, 250 kb, 100 kb, 50 kb, or 20 kb segment of genomic DNA. Alternatively, the first element, second element, and third element may be cointegrated between a left Ti border sequence and a right Ti border sequence.

Alternatively, the transformed plant may comprise a nucleic acid segment containing nucleic acid sequences encoding enzymes in a biosynthetic pathway, where a single promoter directs transcription of the nucleic acid sequences.

The transformed plant may generally be any type of plant, and preferably is a tobacco, wheat, potato, *Arabidopsis*, corn, soybean, canola, oil seed rape, sunflower, flax, peanut, sugarcane, switchgrass, or alfalfa plant.

The promoters may be any of the promoters discussed earlier. The transformed plant preferably produces polyhydroxyalkanoate polymer.

The invention also encompasses methods of preparing transformed host cells. The methods may produce a transformed host cell having nucleic acid sequences under the control of multiple promoters or under the control of a single promoter. The method preferably comprises the steps of selecting a host cell; transforming the selected host cell with a recombinant vector comprising: a first element comprising operatively linked in the 5' to 3' direction: a first promoter that directs transcription of the first nucleic acid sequence; a first nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein; a first 3' transcription terminator; a first 3' polyadenylation signal sequence; a second element comprising operatively linked in the 5' to 3' direction: a second promoter that directs transcription of the second nucleic acid sequence; a second nucleic acid sequence encoding a β-ketoacyl reductase protein; a second 3' transcription terminator; a second 3' polyadenylation signal sequence; and a third element comprising operatively linked in the 5' to 3' direction: a third promoter that directs transcription of the third nucleic acid sequence; a third nucleic acid sequence encoding a β-ketothiolase protein; a third 3' transcription terminator; and a third 3' polyadenylation signal sequence; and obtaining transformed host cells; wherein the transformed host cells produce polyhydroxyalkanoate polymer.

Alternatively, the method of preparing transformed host cells may comprise the steps of selecting a host cell; transforming the selected host cell with a recombinant vector comprising operatively linked in the 5' to 3' direction: a promoter that directs transcription of a first nucleic acid sequence, second nucleic acid sequence, and third nucleic acid sequence; a first nucleic acid sequence; a second nucleic acid sequence; a third nucleic acid sequence; a 3' transcription terminator; and a 3' polyadenylation signal sequence; and obtaining transformed host cells; wherein: the first nucleic acid sequence, second nucleic acid sequence, and third nucleic acid

sequence encode different proteins; the first nucleic acid sequence, second nucleic acid sequence, and third nucleic acid sequence are independently selected from the group consisting of a nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein, a nucleic acid sequence encoding a β-ketoacyl reductase protein, and a nucleic acid sequence encoding a β-ketothiolase protein; and the transformed host cells produce polyhydroxyalkanoate polymer.

The promoters may be any of the promoters discussed earlier.

Also disclosed are methods for preparing transformed plants. The methods may produce a transformed plant having nucleic acid sequences under the control of multiple promoters or under the control of a single promoter. The method preferably comprises the steps of selecting a host plant cell; transforming the selected host plant cell with a recombinant vector comprising: a first element comprising operatively linked in the 5' to 3' direction: a first promoter that directs transcription of a first nucleic acid sequence; a first nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein; a first 3' transcription terminator; and a first 3' polyadenylation signal sequence; a second element comprising operatively linked in the 5' to 3' direction: a second promoter that directs transcription of a second nucleic acid sequence; a second nucleic acid sequence encoding a β-ketoacyl reductase protein; a second 3' transcription terminator; and a second 3' polyadenylation signal sequence; and a third element comprising operatively linked in the 5' to 3' direction: a third promoter that directs transcription of a third nucleic acid sequence; a third nucleic acid sequence encoding a β-ketothiolase protein; a third 3' transcription terminator; and a third 3' polyadenylation signal sequence; obtaining transformed host plant cells; and regenerating the transformed host plant cells to produce transformed plants, wherein the transformed plants produce polyhydroxyalkanoate polymer.

Alternatively, the method of preparing a transformed plant may comprise the steps of selecting a host plant cell; transforming the selected host plant cell with a recombinant vector comprising operatively linked in the 5' to 3' direction: a promoter that directs transcription of a first nucleic acid sequence, second nucleic acid sequence, and third nucleic acid sequence; a first nucleic acid sequence; a second nucleic acid sequence; a third nucleic acid sequence; a 3' transcription terminator; and a 3' polyadenylation signal sequence; obtaining transformed host plant cells; and regenerating the transformed host plant cells to produce transformed plants; wherein: the first nucleic acid sequence, second nucleic acid sequence, and third nucleic acid sequence encode different proteins; the first nucleic acid sequence, second nucleic acid sequence,

and third nucleic acid sequence are independently selected from the group consisting of a nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein, a nucleic acid sequence encoding a β-ketoacyl reductase protein, and a nucleic acid sequence encoding a β-ketothiolase protein; and the transformed plants produce polyhydroxyalkanoate polymer.

The promoters may be any of the promoters discussed earlier.

The invention is also directed towards methods of producing biomolecules of interest. The multiple enzymes in the biosynthetic pathway may lead to the production of materials of commercial and scientific interest. Preferably, the biomolecules are polymers, and more preferably are polyhydroxyalkanoate polymers. The methods may comprise obtaining any of the above described transformed host cells or transformed plants, culturing or growing the transformed host cells or transformed plants under conditions suitable for the production of polyhydroxyalkanoate polymer, and recovering polyhydroxyalkanoate polymer. The methods may further comprise the addition of nutrients, substrates, or other chemical additives to the growth media or soil to facilitate production of polyhydroxyalkanoate polymer. In a preferred embodiment, it is possible to extract the polyhydroxyalkanoate from the transformed host cells or transformed plants without killing the host cells or plants. This may be accomplished, for example, by various solvent extraction methods or by engineering the host cells or plants to secrete the polyhydroxyalkanoate polymer, or by directing production to tissues such as leaves or seeds which may be removed without causing serious injury to the plant. The polyhydroxyalkanoate polymer produced is preferably poly(3-hydroxybutyrate), poly(3-hydroxybutyrate-co-3-hydroxyvalerate), poly(4-hydroxybutyrate), or poly(3-hydroxybutyrate-co-4-hydroxybutyrate).

If repetitive sequences are used in a multi-gene plasmid system, there exists the possibility for gene silencing in subsequent generations of plants. If expression levels are high gene silencing could also occur and would be independent of repetitive elements. Repetitive sequences may include the use of the same promoters, chloroplast peptide encoding sequences, and other genetic elements for each of the multi-gene coding sequences. Gene silencing often manifests itself as a gradual reduction in protein levels, mRNA levels, or biosynthesis product concentrations in subsequent generations of related plants. If gene silencing is observed, changing the repetitive sequences through the use of diverse genetic elements such as different promoters, leaders, introns, transit peptide sequences, etc., different designed nucleotide

sequence, or through mutagenesis of the existing sequence, may be successful in reducing or eliminating the gene silencing effects.

DESCRIPTION OF THE FIGURES

The following figures form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

Figure 1: Biosynthesis of poly(β-hydroxybutyrate-co-β-hydroxyvalerate) (poly(3HB-co-3HV), PHBV) in *Ralstonia eutropha*.

Figure 2: Plant transformation strategies for multi-enzyme metabolic pathway engineering.

Figure 3: Plasmid map of pMON25642. A list of the restriction enzyme cutting sites for pMON25642 is provided in Table 10.

Figure 4: Plasmid map of pMON10098. A list of the restriction enzyme cutting sites for pMON10098 is provided in Table 11.

Figure 5: Plasmid map of pMON969. A list of the restriction enzyme cutting sites for pMON969 is provided in Table 12.

Figure 6: Plasmid map of pMON25661. A list of the restriction enzyme cutting sites for pMON25661 is provided in Table 13.

Figure 7: Plasmid map of pMON25897. A list of the restriction enzyme cutting sites for pMON25897 is provided in Table 14.

Figure 8: Plasmid map of pMON25662. A list of the restriction enzyme cutting sites for pMON25662 is provided in Table 15.

Figure 9: Plasmid map of pMON25663. A list of the restriction enzyme cutting sites for pMON25663 is provided in Table 16.

Figure 10: Plasmid map of pMON25943. A list of the restriction enzyme cutting sites for pMON25943 is provided in Table 17.

Figure 11: Plasmid map of pMON25948. A list of the restriction enzyme cutting sites for pMON25948 is provided in Table 18.

Figure 12: Plasmid map of pMON25949. A list of the restriction enzyme cutting sites for pMON25949 is provided in Table 19.

Figure 13: Plasmid map of pMON25951. A list of the restriction enzyme cutting sites for pMON25951 is provided in Table 20.

Figure 14: Plasmid map of pMON34545. A list of the restriction enzyme cutting sites for pMON34545 is provided in Table 21.

Figure 15: Plasmid map of pMON34565. A list of the restriction enzyme cutting sites for pMON34565 is provided in Table 22.

Figure 16: Plasmid map of pMON25995. A list of the restriction enzyme cutting sites for pMON25995 is provided in Table 23.

Figure 17: Plasmid map of pMON25973. A list of the restriction enzyme cutting sites for pMON25973 is provided in Table 24.

Figure 18: Plasmid map of pMON25987. A list of the restriction enzyme cutting sites for pMON25987 is provided in Table 25.

Figure 19: Plasmid map of pMON25991. A list of the restriction enzyme cutting sites for pMON25991 is provided in Table 26.

Figure 20: Plasmid map of pMON25992. A list of the restriction enzyme cutting sites for pMON25992 is provided in Table 27.

Figure 21: Plasmid map of pMON25993. A list of the restriction enzyme cutting sites for pMON25993 is provided in Table 28.

Figure 22: Plasmid map of pMON36805. A list of the restriction enzyme cutting sites for pMON36805 is provided in Table 29.

Figure 23: Plasmid map of pMON36814. A list of the restriction enzyme cutting sites for pMON36814 is provided in Table 30.

Figure 24: Plasmid map of pMON36816. A list of the restriction enzyme cutting sites for pMON36816 is provided in Table 31.

Figure 25: Plasmid map of pMON36824. A list of the restriction enzyme cutting sites for pMON36824 is provided in Table 32.

Figure 26: Plasmid map of pMON36843. A list of the restriction enzyme cutting sites for pMON36843 is provided in Table 33.

Figure 27: Plasmid map of pMON34543. A list of the restriction enzyme cutting sites for pMON34543 is provided in Table 34.

Figure 28: Plasmid map of pMON36850. A list of the restriction enzyme cutting sites for pMON36850 is provided in Table 35.

Figure 29: Plasmid map of pMON25963. A list of the restriction enzyme cutting sites for pMON25963 is provided in Table 36.

Figure 30: Plasmid map of pMON25965. A list of the restriction enzyme cutting sites for pMON25965 is provided in Table 37.

Figure 31: Method for creating multi-gene vectors.

Figure 32: PHB biosynthetic pathway. PHB production requires the condensation of two acetyl-CoA molecules using a β-ketothiolase, a D-isomer-specific reduction by acetoacetyl-CoA reductase, and PHB polymerization by PHB synthase. The genes encoding these enzymes are indicated in parentheses.

Figure 33: Schematic diagram of multi-gene vector used to transform *Brassica napus*. Vectors were constructed using modular cassettes. Each cassette consists of the *Lesquerella* hydroxylase promoter (P-Lh), a chloroplast transit peptide (ctp) fused to an open reading frame encoding a PHB synthesis enzyme, and the E9 3' terminator. The plasmid also expresses EPSP synthase to provide resistance to glyphosate, contains bacterial replication origins, and a bacterially-expressed gene encoding resistance to streptomycin and spectinomycin. In pMON36814, *bktB* was replaced with *phbA*. Otherwise, the vectors were identical. RB, right border of T-DNA; LB, left border of T-DNA.

Figure 34: Electron micrographs of *Brassica napus* plastids. Panel A: Leukoplast from wild type *Brassica napus* seed. Panel B: Leukoplast from *Brassica napus* seed producing PHB. Polymer (PHB) and oil bodies (O) are indicated. Note the greatly expanded size of leukoplasts in the PHB-producing line.

Figure 35: A pathway designed to produce poly(β-hydroxybutyrate-co-β-hydroxyvalerate) in the plastids of plants. Propionyl-CoA is derived from threonine via threonine deaminase and the pyruvate dehydrogenase complex. Acetyl-CoA is drawn from normal intermediary metabolism. The pathway requires transformation of the plant with four genes (ilvA, bktB, phbB, and phbC), and relies on endogenous

pyruvate dehydrogenase. All enzymes encoded by transgenes are targeted to the plastid using chloroplast transit peptides.

Figure 36: Concentrations of selected 2-keto acids and amino acids in control plants and in Arabidopsis expressing threonine deaminase. (A) Comparison of pyruvate and 2-ketobutyrate concentrations in Arabidopsis harboring either a control plasmid or a plasmid expressing wild type *E. coli* ilvA (threonine deaminase). (B) Comparison of threonine, isoleucine, and 2-ketobutyrate concentrations in *Arabidopsis* harboring either a control plasmid or a plasmid expressing wild type *E. coli* ilvA. Note the different scales used in parts (A) and (B).

Figure 37: ^{13}C NMR spectra demonstrating poly(β-hydroxybutyrate-co-β-hydroxyvalerate) copolymer production in transgenic *Arabidopsis*. Note the presence of signals indicating presence of both 3-hydroxybutyrate and 3-hydroxyvalerate side chains.

Figure 38: Analyses of total polymer production, the 3-hydroxyvalerate fraction of the polymer, and the activity of threonine deaminase *Brassica* oilseeds synthesizing PHBV copolymer. Note the distinct negative correlation between polymer concentration and the 3-HV content of the polymer. Also note that increasing threonine deaminase activity does not lead to increased 3-HV content.

Figure 39: Multiple potential routes to produce propionyl-CoA *in planta*. Most alternative pathways have the potential to produce propionyl-CoA in plants. However, production of propionyl-CoA from threonine provides the most direct route.

Figure 40: Bar graph of average % PHA produced from *Arabidopsis* transformation methods.

Figure 41: Bar graph of average % PHA produced from canola transformation methods.

Figure 42: Bar graph of maximum % PHA produced from *Arabidopsis* transformation methods.

Figure 43: Bar graph of maximum % PHA produced from canola transformation methods.

DEFINITIONS

The following definitions are provided in order to aid those skilled in the art in understanding the detailed description of the present invention.

"Acyl-ACP thioesterase" refers to proteins which catalyze the hydrolysis of acyl-ACP thioesters.

"C-terminal region" refers to the region of a peptide, polypeptide, or protein chain from the middle thereof to the end that carries the amino acid having a free a carboxyl group (the C-terminus).

"CoA" refers to coenzyme A.

The phrases "coding sequence", "open reading frame", and "structural sequence" refer to the region of continuous sequential nucleic acid triplets encoding a protein, polypeptide, or peptide sequence.

The term "encoding DNA" or "encoding nucleic acid" refers to chromosomal nucleic acid, plasmid nucleic acid, cDNA, or synthetic nucleic acid which codes on expression for any of the proteins or fusion proteins discussed herein.

"Fatty acyl hydroxylase" refers to proteins which catalyze the conversion of fatty acids to hydroxylated fatty acids.

The term "genome" as it applies to bacteria encompasses both the chromosome and plasmids within a bacterial host cell. Encoding nucleic acids of the present invention introduced into bacterial host cells can therefore be either chromosomally-integrated or plasmid-localized. The term "genome" as it applies to plant cells encompasses not only chromosomal DNA found within the nucleus, but organelle DNA found within subcellular components of the cell. Nucleic acids of the present invention introduced into plant cells can therefore be either chromosomally-integrated or organelle-localized.

"Identity" refers to the degree of similarity between two nucleic acid or protein sequences. An alignment of the two sequences is performed by a suitable computer program. A widely used and accepted computer program for performing sequence alignments is CLUSTALW v1.6 (Thompson, et al. *Nucl. Acids Res.*, 22: 4673-4680, 1994). The number of matching bases or amino acids is divided by the total number of bases or amino acids, and multiplied by 100 to obtain a percent identity. For example, if two 580 base pair sequences had 145 matched bases, they would be 25 percent identical. If the two compared sequences are of different lengths, the number of matches is divided by the shorter of the two lengths. For example, if there were 100 matched amino acids between 200 and a 400 amino acid proteins,

they are 50 percent identical with respect to the shorter sequence. If the shorter sequence is less than 150 bases or 50 amino acids in length, the number of matches are divided by 150 (for nucleic acids) or 50 (for proteins); and multiplied by 100 to obtain a percent identity.

The terms "microbe" or "microorganism" refer to algae, bacteria, fungi, and protozoa.

"N-terminal region" refers to the region of a peptide, polypeptide, or protein chain from the amino acid having a free a amino group to the middle of the chain.

"Nucleic acid" refers to ribonucleic acid (RNA) and deoxyribonucleic acid (DNA).

A "nucleic acid segment" is a nucleic acid molecule that has been isolated free of total genomic DNA of a particular species, or that has been synthesized. Included with the term "nucleic acid segment" are DNA segments, recombinant vectors, plasmids, cosmids, phagemids, phage, viruses, etcetera.

"Overexpression" refers to the expression of a polypeptide or protein encoded by a DNA introduced into a host cell, wherein said polypeptide or protein is either not normally present in the host cell, or wherein said polypeptide or protein is present in said host cell at a higher level than that normally expressed from the endogenous gene encoding said polypeptide or protein.

The term "plastid" refers to the class of plant cell organelles that includes amyloplasts, chloroplasts, chromoplasts, elaioplasts, eoplasts, etioplasts, leucoplasts, and proplastids. These organelles are self-replicating, and contain what is commonly referred to as the "chloroplast genome," a circular DNA molecule that ranges in size from about 120 to about 217 kb, depending upon the plant species, and which usually contains an inverted repeat region (Fosket, Plant growth and Development, Academic Press, Inc., San Diego, CA, p. 132, 1994).

"Polyadenylation signal" or "polyA signal" refers to a nucleic acid sequence located 3' to a coding region that directs the addition of adenylate nucleotides to the 3' end of the mRNA transcribed from the coding region.

The term "polyhydroxyalkanoate (or PHA) synthase" refers to enzymes that convert hydroxyacyl-CoAs to polyhydroxyalkanoates and free CoA.

The term "promoter" or "promoter region" refers to a nucleic acid sequence, usually found upstream (5') to a coding sequence, that controls expression of the coding sequence by controlling production of messenger RNA (mRNA) by providing the recognition site for RNA polymerase and/or other factors necessary for start of transcription at the correct site. As contemplated herein, a promoter or promoter region includes variations of promoters derived by

means of ligation to various regulatory sequences, random or controlled mutagenesis, and addition or duplication of enhancer sequences. The promoter region disclosed herein, and biologically functional equivalents thereof, are responsible for driving the transcription of coding sequences under their control when introduced into a host as part of a suitable recombinant vector, as demonstrated by its ability to produce mRNA.

"Regeneration" refers to the process of growing a plant from a plant cell (e.g., plant protoplast or explant).

"Transformation" refers to a process of introducing an exogenous nucleic acid sequence (e.g., a vector, recombinant nucleic acid molecule) into a cell or protoplast in which that exogenous nucleic acid is incorporated into a chromosome or is capable of autonomous replication.

A "transformed cell" is a cell whose nucleic acid has been altered by the introduction of an exogenous nucleic acid molecule into that cell.

A "transformed plant" or "transgenic plant" is a plant whose nucleic acid has been altered by the introduction of an exogenous nucleic acid molecule into that plant, or by the introduction of an exogenous nucleic acid molecule into a plant cell from which the plant was regenerated or derived.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLES

EXAMPLE 1: Sources of nucleic acid sequences

Nucleic acid sequences encoding the polyhydroxyalkanoate biosynthetic pathway include: *phbA* and *phbB* (GenBank accession number J04987), *phbC* (GenBank accession

number J05003), and *bktB* (GenBank accession number AF026544). Production of PHBV copolymer can be accomplished by also expressing *E. coli ilvA* (GenBank accession number U00096, overlapping base 3953951:Gruys et al. WO 98/00557). The Ti DNA left border sequence is described in Baker, R.F., et al. (*Plant Mol. Biol.*, 2: 335-350, 1983). The Ti DNA right border sequence is described in Depicker et. al. (*J. Mol. App. Genet.* 1: 561, 1982).

EXAMPLE 2: Analysis of Nawrath *Arabidopsis* plants

Polyhydroxyalkanoates are a form of polyester accumulated by numerous bacterial species as a carbon and energy repository. This class of polymer also has useful thermoplastic properties, and is therefore of interest as a biodegradable plastic. Poly(β-hydroxybutyrate-co-β-hydroxyvalerate) (poly(3HB-co-3HV), PHBV), a form of PHA, is commercially produced via fermentation of *Ralstonia eutropha* (Figure 1). However, it is expected that the cost of production could be dramatically decreased if PHA could be produced in transgenic plants. The first attempts at PHA production in plants utilized transgenic *Arabidopsis* expressing the three genes required for the homopolymer poly-β-hydroxybutyrate (PHB) (Nawrath, C. et al., *Proc. Natl. Acad. Sci. USA.* 91: 12760-12764, 1994). In this work, the authors transformed *Arabidopsis* plants with three independent gene cassettes and crossed the plants using traditional breeding methods. They reported PHB production up to 14% of the cell dry weight. However, this method took a significant amount of time before the three gene pathway could be assembled. In addition, the plants did not maintain a stable *phb*$^+$ phenotype, as determined by our analysis of the progeny of these original plants (Table 1). This problem may be due to co-suppression (Finnegan, J., and D. McElroy. *Bio/Technology.* 12: 883-888, 1994), or to segregation of high-producing insertions in the progeny. The plants produced by Nawrath et al. were not fully characterized genetically, although it is known that all contained multiple insertions of the transgenes.

Table 1. Enzyme activity and polymer data of progeny of Nawrath *Arabidopsis* lines.

		Specific activities		Western results			
plant line	[protein]	thiolase	reductase	PhbA	PhbB	PhbC	% polymer
number	(mg/mL)	(u/mg)	(u/mg)	thiolase	reductase	synthase	(C4)
134	0.158	0.027	0.069	+	+	-	0.041%
140	0.189	0.026	0.019	+	+	-	0.068%
151	0.377	0.042	0.045	+	+	+	0.038%
159	0.127	0.025	0.009	-	-	+	0.053%
168	0.216	0.018	0.034	+	+	+	0.070%
175	0.186	0.010	0.028	+	-	-	0.043%
177	0.166	0.026	0.000	+	-	-	0.043%
203	0.144	0.030	0.043	-	+	+	0.034%
228	0.250	0.038	0.021	+	+	+	0.048%
240	0.192	0.023	0.010	NA	NA	NA	0.045%

EXAMPLE 3: Use of multiple vectors to introduce PHA biosynthesis sequences into *Arabidopsis.*

One vector was constructed containing sequences encoding both acetoacetyl-CoA reductase and PHB synthase proteins. A second vector was constructed containing a sequence encoding a β-ketothiolase protein. Two independent transformation events were obtained corresponding to each of these vectors. The complete pathway was assembled into a single plant using traditional cross-breeding methods. In all cases, plants exhibiting Mendelian segregation consistent with transgene insertion at a single locus were chosen. The results of these experiments are shown in Table 2.

The second strategy pursued was to simultaneously co-transform both plasmids into a single plant (simultaneous co-transformation) and assay the primary transformant for polymer accumulation, or to re-transform plants that already harbored a single vector (serial co-transformation). The results of these experiments are summarized in Table 3. Although the activity of enzymes expressed from the encoding sequences was comparable to that reported by

Nawrath et al., none of the plants generated reached the polymer levels reportedly achieved in their study. Neither their experiments nor these results correlate enzyme activity with the intracellular concentration of PHA polymer (Nawrath, C. et al., *Proc. Natl. Acad. Sci. U.S.A.* 91: 12760-12764, 1994).

Table 2. Polymer data for *Arabidopsis* crosses.

Vector Number	Plant construct description	# of lines assayed	# of lines positive	C4 polymer (% cell dry wt.)
25640	e35s ctpl phbA			0.01 - 1.55%
25665	e35s ctpl phbC	11	10	AVE: 0.651%
	e35s ctpl phbB			SD: 0.596%
25640	e35s ctpl phbA			0.03 - 0.047%
25739	e35s ctpl phbB	20	12	AVE: 0.178%
	e35s ctpl nocC			SD: 0.163%
25785	e35s ctpl bktB			0.04 - 0.88%
25665	e35s ctpl phbC	11	11	AVE: 0.354%
	e35s ctpl phbB			SD: 0.199%
25785	e35s ctpl bktB			0.03 - 0.21%
25739	e35s ctpl phbB	24	9	AVE: 0.065%
	e35s ctpl nocC			SD: 0.053%
25801	e35s ctpl bktB			0.02 - 0.04%
	e35s ctpl ilvA466	8	3	AVE: 0.029%
25665	e35s ctpl phbC			SD: 0.0095%
	e35s ctpl phbB			
25801	e35s ctpl bktB			0.03 - 0.091%
	e35s ctpl ilvA466	17	9	AVE: 0.044%
25739	e35s ctpl phbB			SD: 0.022%
	e35s ctpl nocC			
25812	e35s ctpl bktB			0.03 - 0.102%
	e35s ctpl ilvA w.t.	3	3	AVE: 0.073%
25665	e35s ctpl phbC			SD: 0.035%
	e35s ctpl phbB			
25812	e35s ctpl bktB			0.02 - 0.11%
	e35s ctpl ilvA w.t.	10	7	AVE: 0.064%
25739	e35s ctpl phbB			SD: 0.031%
	e35s ctpl nocC			

64/104 plants positive; AVE = average; SD = standard deviation.

Table 3. Polymer data for re-transformed and co-transformed *Arabidopsis*.

Vector number	Plant construct description	# of lines assayed	# of lines positive	C4 polymer (% cell dry wt.)
25665	e35s ctpl phbC			0.03 - 0.81%
	e35s ctpl phbB	14	6	AVE: 0.25%
RE/25880	e35s ctpl bktB			SD: 0.29%
	e35s ctpl ilvA w.t.			
25665	e35s ctpl phbC			
	e35s ctpl phbB	5	0	NA
RE/25881	e35s ctpl bktB			
	e35s ctpl ilvA219			
25665	e35s ctpl phbC			0.02 - 0.33%
	e35s ctpl phbB	23	4	AVE: 0.16%
RE/25882	e35s ctpl bktB			SD: 1.3%
	e35s ctpl ilvA466			
25785	e35s ctpl bktB			0.02 - 1.67%
25678	e35s ctpl phbB	21	8	AVE: 0.50%
	e35s ctpl phbC			SD: 0.64%
25785	e35s ctpl bktB			0.01 - 0.72%
25740	e35s ctpl phbB	27	18	AVE: 0.11
	e35s ctpl nocC			SD: 0.15
25801	e35s ctpl bktB			0.646 - 0.715%
	e35s ctpl ilvA466	2	1	AVE: 0.681
25678	e35s ctpl phbB			SD: 0.049%
	e35s ctpl phbC			
25801	e35s ctpl bktB			0.02 - 0.17
	e35s ctpl ilvA466	28	16	AVE: 0.083%
25740	e35s ctpl phbB			SD: 0.050%
	e35s ctpl nocC			
25812	e35s ctpl bktB			0.63 - 1.65%
	e35s ctpl ilvA w.t.	3	3 *	AVE: 1.191%
25678	e35s ctpl phbB			SD: 0.463%
	e35s ctpl phbC			
25812	e35s ctpl bktB			0.02 - 0.20%

	e35s ctpl ilvA w.t.	30	9	AVE: 0.112%
25740	e35s ctpl phbB			SD: 0.053%
	e35s ctpl nocC			

64/145 plants positive.
RE indicates that this vector was used to re-transform a plant line.
AVE = average.
SD = standard deviation.

EXAMPLE 4: Construction of multigene vectors for transformation of *Arabidopsis*.

In an attempt to increase the speed and simplicity of genetic analysis, multigene vectors were constructed containing the entire PHB biosynthetic pathway on a single plasmid. Multigene vectors for PHA production in *Arabidopsis* were constructed from a series of base vectors, each with the desired open reading frame under control of the e35s promoter (Odell, J.T., *et al.*, *Nature*, 313: 810-812, 1985) and the E9 3' region (Coruzzi, *EMBO J.* 3:1671-1679, 1984). The first vector in this series, pMON25642 (Figure 3) , harbors *phbC* under control of the e35s promoter in pMON10098 (Figure 4), a vector designed for *Agrobacterium*-mediated transformation of plants. The remaining intermediate vectors are all derived from pMON969 (Figure 5), a high copy-number vector harboring the e35s promoter and the E9 3' region. Constructs derived from pMON969 include those encoding *phbA* (pMON25661; Figure 6), *bktB* (pMON25897; Figure 7), *phbB* (pMON25662; Figure 8), and *ilvA* (pMON25663; Figure 9). From these and similar vectors were derived the final plasmids for transformation of *Arabidopsis*; pMON25943 (Figure 10) pMON25948 (Figure 11), pMON25949 (Figure 12), pMON25951 (Figure 13), and pMON34545 (Figure 14). All cloning procedures were performed using standard ligation techniques (Sambrook, J., *et al.*, "Molecular cloning: A laboratory manual," Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989), except that ligation of *NotI*-cut pMON25949 with the *ilvA*-containing *NotI* restriction fragment of pMON25663 produced plasmid pMON34565 (Figure 15), that serendipitously contained two copies of the *ilvA* fragment. Each copy of *ilvA* contains a SnaBI restriction site, so deletion of a 3155 bp SnaBI restriction fragment from pMON34565 produced plasmid pMON34545, a plasmid with a single copy of *ilvA*.

The final vectors, pMON25943, pMON25948, pMON25949, pMON25951, and pMON34545 were used for *Agrobacterium*-mediated transformation of *Arabidopsis* (Bechtold N., et al. *Comptes Rendus Acad. Sci. Paris Sciences Serie III Sciences de la Vie.* 316: 1194-1199, 1993). This approach has proven successful in generating lines with the highest levels of PHB obtained to date in our laboratory. PHA production in the plants resulting from the first four of these vectors is summarized in Table 4. Data from pMON34545 transformations will be obtained. All of the data in Table 4 were derived from heterozygous plants, and the polymer concentration may increase once the plants are brought to homozygosity. For example, one plant that produced about 7% PHB by dry weight when heterozygous produced polymer up to 13% when homozygous.

Table 4. Polymer results from *Arabidopsis* derived from multigene vectors.

Vector number	Plant construct description	# of lines assayed	# of lines positive	C4 Polymer (% cell dry wt.)
	e35s ctpl phbC			0.11 - 2.94%
25943	e35s ctp2 phbB	34	28	AVE: 1.13%
	e35s ctpl bktB			SD: 0.65%
	e35s ctpl phbC			0.01 - 7.63%
25948	e35s etpl phbA	53	46	AVE: 2.08%
	e35s etpl phbB			SD: 1.56%
	e35s ctpl phbC			0.02 - 7.74%
25949	e35s ctp2 bktB	35	30	AVE: 1.82%
	e35s ctpl phbB			SD: 1.39%
	e35s ctpl phbC			0.20 - 3.78%
25951	e35s ctpl bktB	12	11	AVE: 1.60%
	e35s ctpl phbB			SD: 1.04%

153/172 plants positive for PHB; 7 had greater than 4% dry weight; AVE = average; SD = standard deviation

These results demonstrate that use of a multigene vector provides consistently higher levels of polymer production than were achieved using multiple vectors. The striking beneficial

results in polymer production obtained from the use of multigene vectors are visually displayed in Figures 40 and 42.

There are several possible explanations for the increased levels of polymer present in the multigene vector transformants. One explanation derives from the fact that it was possible to generate more independent lines with the multigene vectors, and the screening of more plants allowed detection of the relatively rare high-producing lines. This is one clear advantage of having the entire pathway on a single vector, but the distribution of polymer production in plants produced by the various methods suggests that numbers alone do not account for the increased polymer production of multigene vectors. It is also possible that having a metabolic pathway genetically linked at a single integration locus is more metabolically favorable due to some level of concerted gene expression and/or mRNA metabolism. This phenomenon is common in bacteria, but there are not many examples of clustering genes in plants for concerted gene expression. Another possibility is that the high local concentration of promoters may lead to locally high levels of transcription factors. Still another possibility is that having the genes tightly linked may reduce gene silencing, or co-suppression, in certain cases.

EXAMPLE 5: Extraction of polymer from *Arabidopsis* and analysis of polymer.

For isolation of polymer from *Arabidopsis*, stems and leaves were harvested and dehydrated by lyophilization for approximately 36 hours. The material was ground to a fine powder, and 100 mg of powder was treated with 10 mL CLOROX bleach (CLOROX is a registered trademark of The Clorox Company, Oakland, CA) for 1 hour with shaking at room temperature. The extract was subjected to centrifugation at 2700 x g for 10 minutes at 4°C, and the supernatant solutions was carefully removed. Ten mL 100% methanol were added, the solution was mixed by vortexing, and then centrifuged again. After a second, identical methanol extraction, the material was allowed to dry overnight. Polymer was extracted from the dried material with 1 mL of chloroform containing 1 µmol/mL methyl-benzoate standard. The tube was heated to 100°C for 2.5 hours, solid material was removed by centrifugation, and the supernatant material was subjected to methanolysis. Methanolysis of polymer and gas chromatographic characterization of the methyl-ester residues were performed as described by Slater et al. (*J. Bacteriol.* 180:1979-1987, 1998).

EXAMPLE 6: Use of multiple vectors for gene expression in the seeds of canola

Production of polyhydroxyalkanoate has also been accomplished within the seed of canola (oil seed rape). Initial efforts followed essentially the same strategy as the initial *Arabidopsis* strategy. That is, one vector carried the sequences encoding acetoacetyl-CoA reductase and PHA synthase proteins, while another carried the sequence encoding a β-ketothiolase protein. However the 7s promoter, which is expressed primarily in the seed, replaced the 35s promoter that was used in the *Arabidopsis* constructs. These 7s promoter vectors were used to transform oilseed rape, homozygous lines were crossed, and PHB accumulation was assayed in the resulting lines (Table 5). A number of lines that produce PHB were identified, but all produced relatively low concentrations of polymer, with the best lines containing about 2% polymer by dry weight.

Table 5. Polymer results for canola crosses.

Vector number	Plant construct description	# of plants assayed	# of plants positive	C4 polymer (% dry wt.)
25638	7s ctpl *phbA*			0.024 - 1.99%
25626	7s ctpl *phbC*	42	37	0.58%
	7s ctpl *phbB*			SD: 0.59%
25638	7s ctpl *phbA*			0.039 -0.053
25741	7s tpss *phbC*	12	2	0.05%
	7s tpss *phbB*			SD: 0.01%
25818	7s ctpl *bktB*			0.04 - 1.67%
	7s ctpl *ilvA w.t.*	22	17	AVE: 0.61%
25626	7s ctpl *phbC*			SD: 0.43%
	7s ctpl *phbB*			
25818	7s ctpl *bktB*			
	7sctpl *ilvA w.t.*	15	0	NA
25741	7s tpss *phbC*			
	7s tpss *phbB*			
25820	7s ctpl *bktB*			0.26 - 0.72%
	7s ctpl *ilvA466*	19	12	AVE: 0.51%
25626	7s ctpl *phbC*			SD: 0.16%
	7s ctpl *phbB*			
25820	7s ctpl *bktB*			
	7s ctpl *ilvA466*	7	0	NA
25741	7s tpss *phbC*			
	7s tpss *phbB*			

EXAMPLE 7: Construction of multigene vectors for transformation of canola

Large vectors for expression of multiple genes have also been used to produce polyhydroxyalkanoate in the seeds of canola (oil seed rape). In this case, the promoter was derived from the fatty acid hydroxylase gene of *Lesquerella* (P-lh) (Broun, P. and C. Somerville. *Plant Physiol.* 113: 933-942, 1997), which is expressed primarily within the developing seed. A series of vectors, each expressing the entire PHA biosynthesis pathway, was used for

transformation of oilseed rape. The multigene vectors were constructed from a series of base vectors, each with the desired open reading frame under control of the *Lesquerella* hydroxylase promoter (P-lh; Broun, P. and Somerville, C.R. *Plant Physiol.*, 113: 933-942, 1987) and the E9 3' region. The first vector in this series, pMON25995 (Figure 16), harbors *phbC* under control of P-lh in pMON25973 (Figure 17), a vector designed for *Agrobacterium*-mediated transformation of plants. The remaining intermediate vectors are all derived from pMON25987 (Figure 18), a high copy-number vector harboring P-lh and the E9 3' region. Constructs derived from pMON25987 (Figure 16) include those encoding *phbA* (pMON25991; Figure 19), *bktB* (pMON25992; Figure 20), *phbB* (pMON25993; Figure 21), and *ilvA* (pMON36805; Figure 22). These intermediate vectors were used to construct the final vectors for oilseed rape transformation; pMON36814 (Figure 23), pMON36816 (Figure 24), and pMON36824 (Figure 25).

Construction of the multigene vectors for oilseed rape was not as straightforward as was the construction of the *Arabidopsis* vectors. This was primarily due to the large size of the promoter (P-lh is about 2.2 kb), and the resulting larger size of the multigene vector intermediates. As the vectors increased in size, it was found to be most efficient to perform ligations of two similar sized fragments, rather than one large vector and one small incoming fragment. In addition, it was desirable to avoid partial digests of the large vectors, and to perform cloning in which opposite ends of an individual fragment were not compatible. A number of intermediate vectors were constructed specifically to allow cloning in this manner. Another advantage of this approach is that it often allowed restriction enzyme-mediated digestion of the parental plasmids prior to transformation of *Escherichia coli* with ligation products. This procedure significantly increased the frequency of correct constructs recovered. The final vectors were used for *Agrobacterium*-mediated transformation of oilseed rape (Fry, J. et al., *Plant Cell Rep.* 6: 321-325, 1987).

The results of oilseed rape transformation with the multigene vectors are shown in Table 6. There are two primary points of interest in these data. First, multigene vectors larger than 26 kb were successfully constructed and used to transform oilseed rape, with a very low percentage of the plants failing to produce polymer. Second, the distribution of polymer concentrations among multigene vector transformants is higher than that of the plants derived from two separate 7s vectors.

Table 6. Polymer results from canola transformed with multigene vectors.

Vector number	Plant construct description	# of plants assayed	# of plants positive	C4 polymer (% dry wt.)
36814	*lhydrox ctp1 phbC*			0.19 - 4.11%
	lhydrox ctp1 phbA	68	59	AVE: 1.43%
	lhydrox tpss phbB			SD: 1.01%
36816	*lhydrox ctpl phbC*	225	195	0.02-6.28%
	lhydrox ctpl bktB			AVE: 1.0%
	lhydrox tpss phbB			SD: 1.02%
36824	*lhydrox ctpl phbC*	185	152	0.10-2.74%
	lhydrox ctpl bktB			AVE: 0.6%
	lhydrox tpss phbB			SD: 0.5%
	lhydrox ctpl ilvA			

The comparative results for PHA production in canola are graphically presented in Figures 41 and 43. The beneficial results obtained from the use of multigene vectors compared to results obtained from traditional methods is visually impressive.

Since the promoters used in these two vectors sets (those containing the 7s promoter and those containing the *Lesquerella* hydroxylase promoter) are different, it cannot be distinguished whether it was the *Lesquerella* promoter or the use of a single vector that led to the increased polymer concentration. However, it is clear that the single vector approach is viable for seed expression of enzymes, including those required for PHA biosynthesis. In addition, the increased speed of plant construction and analysis using a single vector is a clear benefit.

EXAMPLE 8: Extraction of polymer from oilseed rape and analysis of polymer.

For isolation of polymer from canola seed, seeds were ground to a fine powder with a mortar and pestle. Approximately 200 mg of each sample were extracted two times with 10 mL each of hexane for 1 hour at 60°C, then two times with 10 mL each of 100% methanol for one hour at 60°C. This procedure removed oil from the seed. The material was allowed to dry completely overnight. Polymer was extracted from the dried material with 1 mL of chloroform

containing 1 μmol/mL methylbenzoate standard. The tube was heated to 100°C for 5 hours, solid material was removed by centrifugation, and the supernatant material was subjected to methanolysis. Methanolysis of polymer and gas chromatographic characterization of the methyl-ester residues were performed as described by Slater et al. (*J. Bacteriol.* 180: 1979-1987, 1998).

EXAMPLE 9: Multigene vectors for gene expression in monocots

For reasons described above, multigene vectors will also be desirable for expression of multi-enzyme metabolic pathways in monocots. Therefore, vectors designed to produce PHA in the leaves of maize were constructed. These vectors use the e35s, eFMV, or maize chlorophyll A/B binding protein (P-ChlA/B) promoters, and include the HSP70 intron designed to enhance expression in monocots. All enzymes were fused to the *Arabidopsis* RuBisCo small subunit transit peptide. Other promoters might also be used. Examples of vectors designed for gene expression in monocots are pMON36843 (Figure 26), pMON34543 (Figure 27), and pMON36850 (Figure 28). These vectors have been used to transform maize, and polymer was analyzed as described above for *Arabidopsis*. Polymer production is summarized in Table 7.

Table 7. Polymer production in maize using multigene vectors.

Vector number	Plant construct description	# of plants assayed	# of plants positive	C4 polymer (% dry wt.)
36843	*P-e35S phbC*			1.14-4.81%
	P-e35S phbA	93	11	AVE: 1.84%
	P-e35S phbB			SD: 1.04%
34543	*P-eFMV phbC*	34	34	0.15-2.95%
	P-eFMV phbA			AVE: 0.7%
	P-eFMV phbB			SD: 0.9%
36850	*P-ChlA/B, phbC*	132	78	0.1-5.66%
	P-ChlA/B, phbA			AVE: 1.72%
	P-ChlA/B, phbB			SD: 1.17%

EXAMPLE 10: System for construction of large, multigene vectors

Since multigene vectors are optimal for producing high levels of PHB, and this strategy is potentially optimal for expression of other multiple step pathways, a simple method to produce very large, multigene vectors is preferred. Figures 29 and 30 show plasmids pMON25963 and pMON25965, respectively. These vectors, used together, provide a system for constructing very large vectors. Plasmid pMON25965 provides a shuttle vector by which a gene cassette can be cloned into the NotI restriction sites and thereby be flanked by a series of restriction sites. These restriction sites are relatively rare in many genomes, and thereby of utility for subcloning many genes. Plasmid pMON25963 is a binary vector designed for transformation of plants by *Agrobacterium*. It contains a polylinker with the same sites found flanking the NotI restriction sites of plasmid pMON25965. Using this system, a series of gene "cassettes" can be produced using plasmid pMON25965, and each can be sequentially ligated into plasmid pMON25963.

In practice, a series of vectors similar to pMON25965, but having smaller polylinkers, will be preferred. Specifically, this series of vectors would have a single NotI (or similar enzyme) restriction site flanked by one or several other restriction enzyme sites. By ligating cassettes flanked by large portions of the pMON25965 polylinker into pMON25963, relatively large inverted repeats of polylinker DNA are formed. These inverted repeats are unstable in *Escherichia coli*, and plasmids harboring them do not replicate efficiently. Thus, diminishing the size of the polylinker in the shuttle vector can increase the probability of recovering stable recombinants.

Another strategy for generating multigene vectors and reducing the levels of background caused by vector re-ligation is shown in Figure 31. This strategy could be adapted to accommodate any number of enzymes, depending on the availability of unique restriction sites. One can easily design such a polylinker to accommodate one's cloning needs. As the vector becomes larger, one will want to have a larger homologous overlap for the ligation process or choose restriction endonucleases producing ends that are very easily ligated, and not self-compatible. By following the cloning procedure outlined in Figure 31, one can also control the directionality of the clone. If directionality is not important than clones generated from the ligation into the "shuttle vector" in either orientation could be used. (A← C or A → C).

As with any multigene vector strategy, the starting plasmid used for constructing the large multigene plasmids should be taken into consideration. The common plant transformation plasmid pBIN19 (Frisch, D. et al., *Plant Mol Biol* 27: 405-409, 1995) has a starting size of

11,777 bp. In contrast plasmid pMON10098 (Figure 4) has a starting size of 8431bp. The major difference between the two plasmids is the loss of the *trfA* function which is encoded in trans in *Agrobacterium* strain ABI. Providing the *trfA* function in *trans* allows replication only in the specific strains of *Agrobacterium* engineered to harbor *trfA*. It has been shown by Figurski and Helinski (*Proc. Natl. Acad. Sci. U.S.A.* 76: 1648-1652, 1979) that replication factors can function in *trans*. By providing the minimal origins of replication required for maintenance in both *Escherichia coli* and *Agrobacterium* the starting size of the initial plasmid can be reduced significantly.

Other possibilities to reduce the size of the starting plasmid would be to delete *oriT* since this sequence is required for conjugational transfer only. If electroporation is used to introduce the plasmid into *Agrobacterium*, *oriT* is not an essential element. Another possibility would be to use selection that is functional in plants, *Agrobacterium*, and *Escherichia coli*. This could be accomplished by embedding into the plant promoter for the selectable marker a suitable bacterial promoter sequence and a ribosome binding site in proper context with the start codon on the selectable marker. One could also place this selectable marker on the plasmid flanked by its own right and left border sequences. This may allow for the selectable marker to be integrated into the plant chromosome unlinked to the genes of interest and potentially removed from subsequent generations. Alternatively, plants could be co-transformed by taking the multigene plasmid and cotransforming on a separate plasmid the selectable marker for plants. This would eliminate the cloning of the selectable marker on the multi gene plasmid. The selectable marker can be delivered by mixing two different *Agrobacterium* strains, one containing the multigene plasmid and the other containing the selectable marker, or by using the same *Agrobacterium* strain but having different isolates containing either the multi gene plasmid or the selectable marker, or by having the selectable marker coexisting in the same *Agrobacterium* cell with the multigene vector, but on a separate plasmid with a compatible origin of replication.

One can also envision reducing the size of the selectable marker being used by using a *trans* complementation strategy. For example, one could transform a plant with a portion of a NptII gene that expresses a partial protein. If the transformation plasmid carries the complementary portion of the NptII protein, both fragments of the NptII protein may interact to confer resistance to kanamycin. This is analogous to the α-complementation strategy used for creating functional β-galactosidase (reviewed by Zabin, I. *Mol. Cell. Biochem.* 49: 87-96, 1982).

An example of an optimal starting plasmid for engineering multiple genes in plants would contain only the minimal essential elements required for replication in *Escherichia coli* and in *Agrobacterium* (having all other required functions encoded in *trans*) as well as a selection scheme that (1) reduces the need for redundancy in the selectable marker, and/or (2) reduces the size of the selectable marker, or (3) removes the necessity of having the plant selectable marker on the multi gene plasmid. The promoter used for driving the gene of interest in the multi gene vector should consist of the minimal essential elements required for temporal and spatial expression. The termination and polyadenylation signals should also contain only those sequences required for essential function.

EXAMPLE 11: Poly(β-hydroxybutyrate) production in oilseed leukoplasts of *Brassica napus*

Using plants as factories is attractive for the production of biodegradable plastics since current fermentation technology used for the commercial production of polyhydroxyalkanoates (PHA) is prohibitively expensive. The simplest PHA, poly-β-hydroxybutyrate (PHB), has previously been produced in leaves of *Arabidopsis thaliana* (Nawrath, C., et al., *Proc. Natl. Acad. Sci., U.S.A.*, 91: 12760-12764, 1994). *Brassica napus* oilseed, however, may provide a better system for PHB production because acetyl-CoA, the substrate required in the first step of PHB biosynthesis, is prevalent during fatty acid biosynthesis. Three enzymatic activities are needed to synthesize the PHB polymer: a β-ketothiolase, an acetoacetyl-CoA reductase and a PHB synthase. Genes from the bacterium *Ralstonia eutropha* encoding these enzymes were independently engineered behind the seed-specific *Lesquerella fendleri* oleate-12 hydroxylase promoter in a modular fashion. The gene cassettes were sequentially transferred into a single, multi-gene vector which was used to transform *Brassica napus*. PHB accumulated in leukoplasts to levels as high as 7.7% of seed dry weight. Electron microscopy analyses indicate that leukoplasts from these plants are distorted, yet intact, and appear to expand in response to polymer accumulation.

Polyhydroxyalkanoates (PHAs) comprise a class of biodegradable polymers which offer an environmentally-sustainable alternative to petroleum based plastics (reviewed by Poirier, Y., et al., *Biotechnology*, 13: 142-150, 1995). The homopolymer Poly(β-hydroxybutyrate) (PHB), a particularly well studied PHA, is normally synthesized by various species of bacteria under

conditions where nutrients become limited. PHB is stored in granules which can later be mobilized to provide a carbon and energy resource for the bacteria.

One of the best-studied pathways for PHB synthesis is derived from the bacterium *Ralstonia eutropha* (Slater, S.C., et al, *J. Bacteriol.*, 170: 4431-4436, 1988; Schubert, P., et al., *J. Bact.*, 170: 5837-47, 1988; Peoples, O.P., and Sinskey, A.J., *J. Biol. Chem.*, 264: 15298-15303, 1989; Peoples, O.P., and Sinskey, A.J., *J. Biol. Chem.*, 264: 15293-15297, 1989). The pathway requires three enzymes: a β-ketothiolase, an acetoacetyl-CoA reductase, and a PHB synthase (Figure 32). *R. eutropha* uses least two β-ketothiolases, PhbA and BktB (Slater, S.C., et al., *J. Bact.*, 180: 1979-1987, 1998), and both of these enzymes were used in this study. The acetoacetyl-CoA reductase and PHB synthase are designated PhbB and PhbC, respectively (Peoples, O.P., and Sinskey, A.J., *J. Biol. Chem*, 264: 15298-15303, 1989; Peoples, O.P., and Sinskey, A.J., *J. Biol. Chem.*, 264: 15293-15297, 1989).

R. eutropha is fermented commercially for PHA production, but the process is not economically competitive with polymers derived from petroleum. Therefore, novel commercial efforts to produce PHAs focus on using plants as polymer factories. In this respect, our laboratory is considering two model systems: production in leaves and production in seeds. Since acetyl-CoA is a central metabolite for both PHB and fatty acid biosynthesis, and *Brassica napus* seeds are extremely efficient in oil production, the *Brassica* seeds seem an optimal environment in which to produce PHB (U.S. Patent No. 5,502,273). Production of PHB in *Arabidopsis thaliana* leaves has been achieved using *R. eutropha* enzymes (Poirier, Y., et al., *Science*, 256: 520-523, 1992), and additional work showed that polymer accumulation up to 14% of plant dry weight was achieved when the PHB biosynthetic enzymes were targeted to the plastid (Nawrath, C., et al., *Proc. Nat. Acad. Sci.*, 91: 12760-12764, 1994).

The work presented here demonstrates polymer production in the seeds of *Brassica napus* using a multi-gene vector approach. A significant advantage to using these multi-gene vectors is that the entire PHA pathway is introduced simultaneously, thereby obviating the need for elaborate crossing strategies and eliminating the problems associated with insertional effects at multiple loci. Construction of these multi-gene vectors involved the generation of modular cassettes, each harboring an individual gene. The cassettes were then assembled into a single vector expressing the entire PHB biosynthetic pathway (Figure 33). Each cassette consisted of the *Lesquerella fendleri* oleate-12 hydroxylase promoter (Broun, P., et al., *Plant J.*, 13: 201-210,

1998), a chloroplast transit peptide fused to the open reading frame of interest (*bktB, phbA, phbB,* or *phbC*), and the 3' termination region of the *Pisum sativum* rbcSE9 gene (Coruzzi, G., et al., *EMBO J.*, 3: 1671-1679, 1984). The *Lesquerella* promoter contains 2.2 kb of DNA upstream of the coding region for the oleate-12 hydroxylase gene. This promoter was chosen because it is expressed concurrently with the accumulation of storage lipid (Broun, P., et al., *Plant J.*, 13: 201-210, 1998).

Expression of the PHB pathway in *B. napus* was achieved using *Agrobacterium*-mediated transformation, and glyphosate selection was used to identify transgenic events (Fry, J., et al., *Plant Cell Rep.*, 6: 321-325, 1987). The T-DNA transferred into the plants from these experiments exceeded 16 kilobases in size. The co-expression rate of genes from the multi-gene vectors in *Brassica* seeds was high, with 87% of the glyphosate resistant plants also producing polymer. Polymer levels ranged from 0.02-7.7% for the transgenic plants carrying pMON36814 (*R. eutropha phbA, phbB, phbC*) and 0.02-6.3% for those carrying pMON36816 (*R. eutropha bktB, phbB* and *phbC*). The vast majority of plants producing polymer fall within the 0-3.0% polymer range (Table 8) and all polymer-producing lines generated viable seed.

Table 8. Polymer results from canola multigene vector transformations.

Vector	Genetic elements	# of plants assayed	# of plants positive	C4 polymer (% dry wt.)
	p-Lh, phbC			0.02% - 7.68%
36814	*p-Lh, phbA*	208	180	Avg: 1.73%
	p-Lh, phbB			SD: 1.45%
	p-Lh, phbC			0.02% - 6.28%
36816	*p-Lh, bktB*	225	195	Avg: 1.00%
	p-Lh, phbB			SD: 1.02%

The *B. napus* line displaying 7.7% polymer was further analyzed by electron microscopy. Micrographs revealed that polymer accumulated within the plastid (Figure 34), and that essentially every plastid contained polymer. Polymer production in the plastids is seemingly well tolerated; the size of the plastid expands to accommodate polymer production (compare Figures 34A and 34B). This phenomenon is similar to the size changes observed when amyloplasts accumulate starch, and suggests that plastids will change size to accommodate accumulation of any granular product. Thus, the signal initiating an increase in plastid volume is

not specifically linked to accumulation of normal metabolites; rather, the increase is probably initiated simply by physical pressure applied to the plastid membrane.

These results demonstrate that PHA accumulation is possible in an oilseed system. Commercial oilseed PHA production will require approximately twice the amount of PHA accumulation achieved here. Moreover, commercial success will rely on the development of an integrated processing system to extract PHA, oil, and meal from the seeds. We believe that increases in PHA accumulation can be obtained using alternative promoters that are stronger and expressed for a longer duration during seed development. Other concerns regarding the feasibility of PHA production *in planta* largely revolve around the metabolic effects of PHA production in oilseeds. Specifically, analysis of the effect of PHA production on oil yield will be of particular interest, since both are derived from acetyl-CoA and produced simultaneously. Any untoward effect of PHA production on oil yield or seed quality will impact negatively on the economic feasibility of using *B. napus* as a commercial system.

Vector Construction and Plant Transformation

A single vector encoding the entire PHB biosynthetic pathway was used for *Agrobacterium*-mediated transformation of *Brassica*. This vector, pMON36814, encodes *bktB, phbB,* and *phbC* (Figure 33). Each gene of interest was fused to a chloroplast transit peptide (ctp), so each protein is transported to the seed leukoplast. All enzymes were fused to the *Arabidopsis* RuBisCo small subunit 1a transit peptide that was previously used for PHB production (Nawrath, C, et al., *Proc. Nat. Acad. Sci.,* 91: 12760-12764, 1994) except PhbB was fused to the transit peptide from pea RuBisCo small subunit (Cashmore, A.R., eds. Kosuge, T., Meredith C.P., Hollaender, A., (Plenum, New York), 29-38, 1983). Each gene is controlled by the promoter from the fatty acid hydroxylase gene of *Lesquerella* (P-Lh; Broun, P., et al., *Plant J.*, 13: 201-210, 1998), and terminated with the E9 3' region of the *Pisum* rbcSE9 gene (Coruzzi, G., et al., *EMBO J.*, 3: 1671-1679, 1984). P-Lh directs expression of these genes within the developing seed. The selection cassette for pMON36812 and 36814 consisted of the Figwort Mosaic Virus promoter followed by the *Petunia* RuBisCo small subunit 1a transit peptide, the *Petunia* EPSP synthase gene (CP4) and nopaline synthase 3' termination/polyadenylation region (nos3').

Transformation of *Brassica napus* was done as described in Fry, J. et al. (*Plant Cell Rep.*, 6: 321-325, 1987) using glyphosate for selection.

Polymer Analysis

For isolation of polymer from canola seed, seeds were ground to a fine powder with a mortar and pestle. Approximately 200 mg of each sample were extracted two times in a glass tube with 10 mL each of hexane for 1 hour at 60°C, then two times with 10 mL each of 100% methanol for one hour at 60°C. This procedure removes oil from the seed. The material was allowed to dry completely overnight. Polymer was extracted from the dried material with 1 mL of chloroform containing 3 μmol/mL methyl-benzoate standard. The tube was heated to 100°C for 5 hours and the samples were cooled. One mL methanol/sulphuric acid (85:15, v/v) was added, and the mixture was heated to 100°C for exactly 2.5 hours. The solution was cooled, extracted with water and subjected to gas chromatography. Gas chromatographic characterization of the methyl-ester residues was performed as described by (Slater, S., et al., *J. Bact.*, 180: 1979-1987, 1998) except that the temperature gradient was performed as follows: the initial temperature of 70°C was held for 6 minutes, then the temperature was increased by 30°C per minute to 130°C. Finally, the temperature was increased by 50°C per minute to 300°C and held at 300°C for 5 minutes.

Electron Microscopy:

Partial imbibition of *Brassica* seeds was achieved by the slight abrasion of the seed coats, followed by placement for 2 hours onto filter paper moistened with distilled water. The cotyledons of these seeds were then cut into 1 mm^3 pieces and fixed in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 for three hours, with the first 30 minutes under vacuum. The tissue was post-fixed in 1% osmium tetroxide in the above buffer, dehydrated in ethanol and propylene oxide and infiltrated with a 1:1 mixture of Spurr's: EMbed 812 resin. The resin was polymerized at 60°C for 48 hours. The resulting blocks were sectioned on an Leica Ultracut E microtome. Sections 80 nm thick were picked up on formvar/carbon coated copper slot grids. The grids were post-stained with uranyl acetate and lead citrate in an LKB ultrastainer and

examined with a JEOL 1200 transmission electron microscope. (All reagents were obtained from Electron Microscopy Sciences, Fort Washington, PA).

EXAMPLE 12: Metabolic Engineering of *Arabidopsis* and *Brassica* for poly(β-hydroxybutyrate-*co*-β-hydroxyvalerate) copolymer production

Poly(hydroxyalkanoates) are natural polymers with thermoplastic properties. One polymer of this class, poly(β-hydroxybutyrate-co-β-hydroxyvalerate) (PHBV) is currently produced by bacterial fermentation, but the process is not economically competitive with polymer production from petrochemicals. PHA production in green plants promises much lower costs, but producing polymer with the appropriate monomer composition is problematic. By redirecting metabolic pools of both short-chain fatty acids and amino acids, *Arabidopsis* and *Brassica* have now been engineered to produce PHBV, a copolymer with commercial applicability. In this Example, polymer production, metabolic intermediate analyses, and pathway dynamics for PHBV synthesis *in planta* are described.

Poly(hydroxyalkanoates) (PHAs) are a class of polymers accumulated by numerous bacterial species as carbon and energy reserves. These polymers have thermoplastic properties, and have received much attention as biodegradable alternatives to petrochemical plastics (Anderson, A. J., and Dawes, E. A. *Microbiol. Rev.* 54: 450-472, 1990). While the homopolymer poly(β-hydroxybutyrate) (PHB) is somewhat brittle, many copolymers such as poly(β-hydroxybutyrate-co-β-hydroxyvalerate) (PHBV) are more flexible due to reduced crystallinity, and suitable for many commercial applications.

The biochemical pathways for PHB and PHBV production are essentially identical, differing only in the initial metabolites. PHB synthesis is initiated by condensation of two acetyl-CoA molecules, whereas PHBV synthesis requires the additional condensation of acetyl-CoA with propionyl-CoA. Following condensation, the products are reduced by a D-isomer specific acetoacetyl-CoA reductase, and the resulting β-hydroxy products are polymerized by PHB synthase (Anderson, A. J., and Dawes, E. A. *Microbiol. Rev.* 54: 450-472, 1990; Steinbüchel and Schlegel, *Mol. Microbiol.* 5(3):535-42, 1991).

PHBV is produced commercially by growing *Ralstonia eutropha* on glucose and propionate (Byrum, D. *FEMS Microbiol. Rev.* 102: 247-250, 1992), but the cost of this process

prohibits large-scale fermentation. Production of PHAs via genetic engineering of green plants is expected to reduce costs to economical levels (van der Leij, F. R., and Witholt, B. *Can. J. Microbiol.* 41(Suppl.1): 222-238, 1995), and production of PHB homopolymer in plants has been demonstrated (Poirier, Y., et al. *Science* 256: 520-523, 1992; Nawrath, C.; et al. *Proc. Natl. Acad. Sci.* 91: 12760-12764, 1994). However copolymer production has been problematic, primarily due to the requirement for metabolic precursors other than acetyl-CoA.

Here we report metabolic engineering of plants to produce PHBV copolymer. By expressing four distinct transgenes and diverting metabolic pools of acetyl-CoA and threonine, copolymer was produced in *Arabidopsis thaliana,* and in the seeds of *Brassica napus* (oilseed rape). PHBV copolymer production opens the use of green plants as factories for commercial, environmentally-sustainable production of biodegradable plastics.

Results: A Pathway for Poly(β-hydroxybutyrate-co-β-hydroxyvalerate) Production in Plants.

A pathway designed to engineer PHBV production in the plastids of plants is diagrammed in Figure 35. Acetyl-CoA is drawn from plastid intermediary metabolism, whereas propionyl-CoA is generated from threonine *via* 2-ketobutyrate (Gruys et al WO 98/00557; Eschenlauer, A.C., et al. *Int. J. Biol. Macromol.* 19: 121-130, 1996). This pathway requires transformation of the plant with four separate genes: *ilvA, bktB, phbB,* and *phbC*. It also relies on the endogenous plastid pyruvate dehydrogenase complex (PDC). The threonine deaminase used in these studies is the biosynthetic enzyme IlvA from *E. coli* (Taillon, B.E., et al. *Gene* 63: 245-252, 1988). The acetoacetyl-CoA reductase (PhbB) and PHB synthase (PhbC) are the same *R. eutropha* enzymes used in earlier *in planta* studies (Poirier, Y., et al. *Science* 256: 520-523, 1992; Nawrath, C.; et al. *Proc. Natl. Acad. Sci.* 91: 12760-12764, 1994). The β-ketothiolase is BktB from *R. eutropha* (Slater, S., et al. *J. Bacteriol.* 180: 1979-1987, 1998). Previous work on PHB production in plants used the *R. eutropha* PhbA β-ketothiolase. However, PhbA cannot efficiently synthesize β-ketovaleryl-CoA, whereas BktB produces both β-ketovaleryl-CoA and acetoacetyl-CoA.

Metabolic Engineering of *Arabidopsis* and *Brassica*.

Polymer production was studied in both *Arabidopsis thaliana* leaves and *Brassica napus* seeds. For PHBV production in *Arabidopsis*, two separate vectors were constructed. Plasmid pMON25678 encodes *phbB* and *phbC*, and plasmid pMON25812 encodes *bktB* and *ilvA*. Transgenic *Arabidopsis* were generated by simultaneous *Agrobacterium*-mediated transformation with both vectors, and subsequent selection on both glyphosate and kanamycin. All genes were controlled by the e35S promoter (Odell, J.T., et al. *Nature* 313: 810-812, 1985), leading to polymer production throughout the plant. In *Brassica*, all four genes in the transgenic pathway were expressed from a single vector, pMON36824, and polymer production was directed to the seeds by the *Lesquerella* hydroxylase promoter (Broun, P., et al. *Plant J.* 13: 201-210, 1998).

Previous work on PHA production in plants has shown that polymer is produced efficiently and that phenotypic effects on the plant are minimized when PHA production occurs in the chloroplasts (Nawrath, C. et al. *Proc. Natl. Acad. Sci.* 91: 12760-12764, 1994). The plastids are the site for synthesis of both oil, which is derived from acetyl-CoA, and threonine which is used to produce propionyl-CoA. In both *Arabidopsis* and *Brassica*, the PHA biosynthesis enzymes were targeted to the plastids using chloroplast transit peptides. In photosynthetic tissues of *Arabidopsis* the proteins are targeted to the chloroplasts, whereas in *Brassica* seeds the enzymes are targeted to the leucoplasts.

Generation of Propionyl-CoA from Threonine.

Conversion of threonine to 2-ketobutyrate by IlvA is the first reaction catalyzed by one of the recombinantly-encoded enzymes. IlvA normally catalyzes the initial step in the conversion of threonine to isoleucine, and the enzyme is feedback-inhibited by isoleucine (Umbarger, H.E. Biosynthesis of branched-chain amino acids, pp. 442-457 in Escherichia coli and Salmonella: Cellular and Molecular Biology, Neidhart, F.C., Curtiss, R., Lin, E.C.C., Low, K.B., Magasanik, B., Reznikoff, W.S., Riley, M., Schaechter, M., and Umbarger, H.E. (eds.).ASM Press, Washington, D. C., 1996). However, *ilvA* mutants with diminished sensitivity to isoleucine have been described and two such mutants, *ilvA*466 (Pledger, W.J.. and Umbarger, H.E. *J.*

Bacteriol. 114: 183-194, 1973; Taillon, B.E., et al. *Gene* 63: 245-252, 1988) and *ilvA219* (Burns, R.O., et al. *J. Biol. Chem.* 254: 1074-1079, 1979; Eisenstein, E., et al. *Biochemistry* 34: 9403-9412, 1995), were used along with wild-type *ilvA* in these studies. IlvA466 is partially sensitive to feedback inhibition by isoleucine, and IlvA219 is essentially insensitive (Pledger, W.J., and Umbarger, H.E. *J. Bacteriol.* 114: 195-207, 1973; LaRossa, R.A., et al. *J. Bacteriol.* 169: 1372-1378, 1987).

Both *Arabidopsis* and *Brassica* were initially transformed with separate vectors expressing wild-type *ilvA*, *ilvA466*, and *ilvA219*. In both organisms, no fertile transformants expressing *ilvA219* were recovered, indicating that expression of completely isoleucine-insensitive IlvA is lethal. In *Arabidopsis*, plants expressing *ilvA466* were recovered at a very low frequency, whereas *Brassica* tolerated *ilvA466* rather well. This result may be due to the seed-specific nature of the *Lesquerella* promoter. Transformants expressing wild-type *ilvA* were efficiently recovered in both *Arabidopsis* and *Brassica*.

In order to monitor the metabolic effects of IlvA in transgenic plants, metabolites likely to be effected by this enzyme were analyzed. Figure 36 shows profiles of selected 2-ketoacids and amino acids in a control plant, and in transgenic *Arabidopsis* expressing wild-type *ilvA*. As expected, the transgenic plant had elevated levels of both 2-ketobutyrate and isoleucine. In addition, a high concentration of 2-aminobutyrate was present. Formation of 2-aminobutyrate from 2-ketobutyrate is a freely-reversible reaction, probably catalyzed by the same branched-chain amino acid transaminase that catalyzes the final step in isoleucine biosynthesis (Singh, B.K. (1999) Biosynthesis of Valine, Leucine and Isoleucine. In: Singh, B.K. (ed.) Plant Amino Acids: Biochemistry and Biotechnology. Marcel Dekker, Inc., New York, pp.227-247, 1998). Although transgenic plants expressing *ilvA* contained more 2-ketobutyrate than did wild-type plants, the 2-ketobutyrate concentration was still below that of pyruvate. Most 2-ketobutyrate was apparently diverted to produce 2-aminobutyrate and isoleucine. The concentration of free threonine in a plant expressing *ilvA* decreased by only about 15%, suggesting that threonine synthesis was sufficiently robust to compensate for the diversion of threonine through 2-ketobutyrate. Similar analyses were performed on the seeds from control and transgenic *Brassica*, and essentially the same results were obtained. In plants expressing *ilvA*, isoleucine, 2-ketobutyrate, and 2-aminobutyrate concentrations were elevated, and free threonine was only marginally decreased (K. Gruys et al., unpublished data).

The second step in the formation of propionyl-CoA is catalyzed by the plastid pyruvate dehydrogenase complex, which is the sole endogenous enzyme required for PHBV production. This enzyme complex normally plays a central role in metabolism by converting pyruvate to acetyl-CoA. We found that PDC from isolated *Brassica* leukoplasts was also capable of converting 2-ketobutyrate to propionyl-CoA. However, PDC was approximately 10-fold less efficient when utilizing 2-ketobutyrate than when utilizing pyruvate; the specific activities were 0.4 units/mg and 3.6 units/mg for 2-ketobutyrate and pyruvate, respectively.

Synthesis of PHBV Copolymer.

Once propionyl-CoA has been produced, the pathway is identical to that shown to produce PHBV copolymer in recombinant *E. coli* (Slater, S., et al. *J. Bacteriol.* 180: 1979-1987, 1998). Propionyl-CoA is converted to D-β-hydroxyvaleryl-CoA by BktB and PhbB, and then is polymerized with D-β-hydroxybutyryl-CoA to form PHBV copolymer. The functionality of the entire pathway in plants is shown in Figure 37, which shows ^{1}H-NMR spectra demonstrating the presence of PHBV copolymer in *Arabidopsis*. We also obtained ^{13}C-NMR demonstrating PHBV copolymer production in *Brassica*, and all these data have been corroborated by coupled gas chromatography-mass spectrometry (data not shown). The molecular weight of PHBV isolated from *Brassica* seeds was approximately 1 x 10^{6}, with a polydispersity index of 2.4. These parameters are suitable for commercial applications.

Although copolymer was made in both *Arabidopsis* and *Brassica*, the 3-hydroxyvalerate component varied with the *in vivo* polymer concentration. The polymer composition in *Brassica* seeds distinctly showed a negative correlation between the 3-hydroxyvalerate content of the polymer and total polymer production (Figure 38). Threonine deaminase activity also negatively correlated with 3-HV content (Figure 38), a somewhat surprising result considering the role of IlvA in the production of 3-HV. However, we have consistently found that introduction of vectors encoding multiple genes leads to a general, concerted expression of all encoded enzymes. Thus, elevated IlvA activity is consistent with elevated polymer production.

Discussion

The use of green plants as industrial factories will often require significant changes in plant metabolism, so metabolic engineering of multi-step pathways will become an important technology in "green chemistry" efforts. In this study, production of the PHA copolymer PHBV has been accomplished using a combination of endogenous and transgene-encoded enzymes. The pathway consists of five separate enzymes, four being encoded as transgenes. In the case of *Brassica*, all four genes were successfully introduced on a single vector.

Commercial application of this technology will rest on two primary metabolic issues: 1) can polymer be produced *in planta* to concentrations amenable to economical polymer extraction? and 2) as the polymer concentration increases, can the appropriate monomer composition be maintained? We expect that polymer concentrations *in planta* will need to reach at least 15% of dry weight for economical production to be feasible. PHB homopolymer concentrations near 15% have been reported (Nawrath, C. et al. *Proc. Natl. Acad. Sci.* 91: 12760-12764, 1994) and have also been achieved in our laboratory (data not shown). Thus, high-level PHB production appears technically attainable.

Production of PHBV copolymer has been accomplished in this study, although all plants produced copolymer at levels below 3% of plant tissue dry weight. The next challenge is high-level production of copolymer, and the data in Figure 38 show that additional work is required to maintain the 3-hydroxyvalerate composition at high polymer concentrations. Specifically, as polymer production increased, the 3-hydroxyvalerate fraction of the polymer decreased, and increasing threonine deaminase expression did not effect this correlation. These data suggest a metabolic bottleneck in the provision of 3-hydroxyvalerate to PHA synthase. The BktB, PhbB, PhbC pathway efficiently synthesizes PHBV copolymer (Slater, S., et al. *J. Bacteriol.* 180: 1979-1987, 1998), and production of 2-ketobutyrate *in planta* is efficient, as estimated from the elevated levels of 2-ketobutyrate, 2-aminobutyrate and isoleucine (Figure 36). Thus, the metabolic bottleneck must exist at the conversion of 2-ketobutyrate to propionyl-CoA by the pyruvate dehydrogenase complex. As noted above, the PDC strongly prefers pyruvate as a substrate, and this difference is compounded *in vivo* by the concentration ratio of pyruvate to 2-ketobutyrate (Figure 36). Pyruvate dehydrogenase apparently cannot effectively compete for 2-ketobutyrate so propionyl-CoA synthesis is limited..

Production of copolymer to high internal concentrations may require a supplementary route for conversion of 2-ketobutyrate to propionyl-CoA. There are several ways to bypass the

PDC or supplement its activity, but all will require additional transgenes. These routes include modifying the α-ketoacid dehydrogenase to more readily accept propionyl-CoA (Inoue H, et al. *J Bacteriol.* 179: 3956-3962, 1997; Gruys et al WO 98/00557), expression of an alternative enzyme complex capable of forming propionyl-CoA from 2-ketobutyrate (Kerscher, L. and Oesterhelt, D., *Eur. J. Biochem.* 116: 587-594, 1981), or co-expression of a propionyl-CoA dehydrogenase (Horswill et al; Mitsky et al., unpublished data) with a propionyl-CoA synthetase or CoA transferase (Gruys et al WO 98/00557; Valentin et al, manuscript in preparation). Thus, a commercially viable transgenic plant producing PHA polymer from threonine may contain up to six separate transgenes.

Synthesis of propionyl-CoA can also be achieved through other metabolic pathways, although none presents a straightforward alternative to the threonine derived pathway (Figure 39). For instance, propionyl-CoA may be generated from acetyl-CoA using a 5-step pathway, part of which is involved in propionyl-CoA degradation in plants (Goodwin, T.W. and Mercer, E.I. Introduction to Plant Biochemistry. Second Edition. Pergamon Press, Oxford, 1985; Eisenreich, W., et al. *Eur. J. Biochem.* 215: 619-632, 1993; Preifert, H., and Steinbüchel, A. *J. Bacteriol.* 174: 6590-6599, 1992; Podkowinski, J., et al. *Proc. Natl. Acad. Sci. USA* 93: 1870-1874, 1996; Sun, J., et al. *Plant Physiol.* 115: 1371-1383, 1997; Horswill A.R., and Escalante-Semerena J.C. *J. Bacteriol.* 179: 928-940, 1997; Gruys et al, unpublished data). Conversion of acrylyl-CoA to propionyl-CoA is potentially problematic, but an appropriate enzyme may be available from *Chroroflexus aurantiacus* (Eisenreich, W., et al. *Eur. J. Biochem.* 215: 619-632, 1993). Propionyl-CoA can also be derived from succinyl-CoA using a pathway present in both *Rhodococcus ruber* and *Nocardia corallina* (Williams, D.R.,et al. *Appl. Microbiol. Biotechnol.* 40: 717-723, 1994; Valentin, H.E., and Dennis, D. *Appl. Environ. Microbiol.* 62: 372-379, 1996). This pathway is initiated by methylmalonyl-CoA mutase, an enzyme that requires vitamin B_{12} as a cofactor. However, vitamin B_{12} is not synthesized in plants (Goodwin, T.W. and Mercer, E.I. Introduction to Plant Biochemistry. Second Edition. Pergamon Press, Oxford, 1985). *Rhodococcus* and *Nocardia* also produce minor amounts of 3-hydroxyvaleryl-CoA via a different, uncharacterized route. This route may be a link to amino acid metabolism, such as the pathways used by other bacteria and animals to degrade valine and isoleucine (Figure 39). These pathways might also be engineered in plants, but a large number of genes are required.

Several other amino acids can be used to produce propionyl-CoA. Methionine, like threonine, generates 2-ketobutyrate during catabolism. This conversion is catalyzed by L-methionine γ-lyase in a reaction that also produces ammonia and methanethiol (Tanaka, H., et al. *Enzyme Microb. Technol.* 7: 530-537, 1985). The effect of methanethiol production on plants is unknown, and supplementation of PDC activity would still be required to efficiently produce propionyl-CoA. Another pathway, present in *Clostridium propionicum*, converts alanine to propionyl-CoA via lactic acid, lactyl-CoA and acrylyl-CoA (Schweiger, G., and Buckel, W. *FEBS Lett.* 171: 79-84, 1984; Cardon, B. P., and Barker, H. A. *Arch. Biochem. Biophys.* 12: 165-180, 1947). However, none of the required genes has been cloned, and some of the necessary enzymes are oxygen sensitive (Hofmeister, A.E.M., and Buckel, W. *Eur. J. Biochem.* 206: 547-552, 1992; Kuchta, R.D., and Abeles, R.H. *J. Biol. Chem.* 260: 13181-13189, 1985). β-alanine is another potential starting metabolite for the production of propionyl-CoA (Arst, H.N. Jr. *Mol. Gen. Genet.* 163: 23-27, 1978; Roberts, E., and Bregoff, H.M. *J. Biol. Chem.* 201: 393-398, 1953; Kupiecki, R.P., and Coon, M.J. *J. Biol. Chem.* 229: 743-754, 1957). β-alanine normally plays a critical role as a precursor to Coenzyme-A and acyl carrier protein. However, little is known about the concentration and compartmentalization of β-alanine in plants, and propionyl-CoA may actually be required for its synthesis.

In summary, poly(β-hydroxybutyrate-co-β-hydroxyvalerate) copolymer was produced in both *Arabidopsis* and *Brassica* by simultaneously accessing amino acid and short-chain fatty acid metabolite pools. In *Brassica*, all four required transgenes were introduced on a single vector, eliminating the plant crossing normally necessary to assemble a pathway of this size. The polymer molecular mass was adequate for commercial purposes, but an apparent metabolic bottleneck in conversion of 2-ketobutyrate to propionyl-CoA suggests that additional engineering may be required to achieve high-level production of polymer with the necessary β-hydroxyvalerate composition.

Generation of ilvA mutants.

All *ilvA* alleles used herein are derived from the *E. coli ilvA* gene (Lawther, R.P. et al., *Nucl. Acids Res.* 11: 2137-2155, 1987) that is harbored in pMON25659 (Gruys et al WO 98/00557). The *ilvA*219 mutation (Eisenstein, E., et al. *Biochemistry.* 34: 9403-9412, 1995) and

*ilvA*466 mutation (Taillon, B. E., et al. *Gene*. 63: 245-252, 1988), both originally isolated in *Salmonella typhimurium*, were introduced into the *E. coli* gene by oligonucleotide-directed mutagenesis as previously described (Gruys et al. WO 98/00557).

Plasmid Construction and Transformation of *Arabidopsis thaliana* and *Brassica napus*

All transformation vectors are derived from pMON10098, a vector designed for *Agrobacterium*-mediated transformation of plants that encodes the nptII selectable marker. The *trfA* function is provided in *trans* by the host bacterium, *Agrobacterium tumefaciens* ABI. *A. tumefaciens* ABI is *Agrobacterium* strain GV3101 (Van Larebeke, N., et al. *Nature*. 252: 169-170, 1974) harboring the helper plasmid pMP90RK (Koncz, C., and Schell, J. *Mol. Gen. Genet.* 204: 383-396, 1986).

All PHA production genes used in this study were initially constructed in intermediate vectors as cassettes including a promoter, a chloroplast transit peptide fused to the gene of interest, and a 3' control region. In every case, the gene cassette is flanked by Not I restriction sites, plus several additional unique restriction sites. Each cassette was excised from it's intermediate vector using appropriate restriction enzymes, and sequentially ligated into the recombinant vector for plant transformation.

For metabolite analysis, *Arabidopsis* was transformed with either pMON15715, an *ilvA*-negative control vector, or pMON25668, which expresses both *phbA* and wild-type *ilvA* from e35S promoters.

For production of PHBV in *Arabidopsis*, two separate plasmids were used.

The first vector encoded both *phbB* and *phbC* (pMON25678), and the second vector encoded both *bktB* and *ilvA* (pMON25812). All genes were controlled by the e35S promoter (Odell, J. T., et al. *Nature*. 313: 810-812, 1995) and the E9 3' region (Coruzzi, G., et al. *EMBO J.* 3: 1671-1679, 1984). All enzymes were fused to the *Arabidopsis* RuBisCo small subunit 1a transit peptide that was previously used for PHB production (Nawrath, C., et al. *Proc. Natl. Acad. Sci.* 91: 12760-12764, 1994). Plasmid pMON25678 encodes resistance to glyphosate, whereas pMON25812 encodes resistance to kanamycin. Both plasmids were simultaneously used for *Agrobacterium*-mediated *Arabidopsis* transformation (Bechtold N., et al. *Comptes*

Rendus Acad. Sci. Paris Sciences Serie III Sciences de la Vie. 316: 1194-1199, 1993), and transformants were selected on both glyphosate and kanamycin as follows.

Arabidopsis thaliana Columbia plants were grown in Metro Mix 200 in 2.5 in. pots covered with a mesh screen. Sown seed was vernalized for 5 days and germinated under conditions of 16 hours light /8 hours dark at 20°C to 22°C, 75% humidity. Plants were watered and fertilized twice weekly with ½X Peters 20-20-20 until infiltration.

A 1:50 dilution of an overnight culture of *Agrobacterium tumefaciens* ABI strain was grown at 28°C in YEP containing Spectinomycin 100 mg/L, Streptomycin, 100 mg/L, Chloramphenicol 25 mg/L, and Kanamycin 50 mg/L. Each culture contained a different ABI construct. After 16-20 hours the *Agrobacterium* cultures were concentrated by centrifugation. The supernatant was discarded and the cell pellets were dried and resuspended in infiltration medium (MS Basal Salts 0.5%, Gamborg's B-5 Vitamins 1%, Sucrose 5%, MES 0.5 g/L, pH 5.7) with 0.44 nM benzylaminopurine (10 µL of a 1.0 mg/L stock in DMSO per liter) and 0.02% Silwet L-77 to an OD_{600} of 0.8. For co-infiltrations each culture was resuspended as described above and 150 mL each of two cultures were combined for a total of 300 mL.

Plants were soaked in water 30 minutes prior to infiltration. Inverted plants were placed into the cultures and vacuum infiltrated at 27 in. Hg for 10 minutes. The plants were placed on their sides in a diaper-lined tray and covered with a germination dome for one day. The pots were then turned upright and were not watered for five days. Infiltrated plants were grown to maturity as described above. Ripe seeds were harvested and sterilized. Harvested seed was placed in a 15 mL Corning tube and sterilized. The tubes containing seed were placed on their sides with lids loosened in a vacuum dessicator containing a beaker of Clorox and 1:100 hydrochloric acid. The dessicator was then sealed with a vacuum and the seed remained in the dessicator overnight. Sterilized seeds from co-infiltrated plants were placed on media containing MS Basal Salts 4.3 g/L, Gamborg's B-5 (500 X) 2.0 g/L, glucose 10 g/L, MES 0.5 g/L, and 8 g/L phytagar with carbenicillin 250 mg/L, cefotaxime 100 mg/L, kanamycin 60 mg/L and 4 mM glyphosate. The seed was germinated at 26°C, 20 hours light / 4 hours dark. Transformants were transferred to soil and covered with a germination dome for one week. The plants were grown in plant growth conditions described above.

For transformation of *Brassica napus*, a single vector encoding the entire PHBV biosynthesis pathway was used. This vector, pMON36824, encodes *bktB, phbB, phbC,* and

ilvA466 (Figure 3). As with the *Arabidopsis* vectors, each gene of interest was fused to a chloroplast transit peptide, so each protein is transported to the seed leukoplast. All enzymes were fused to the *Arabidopsis* RuBisCo small subunit 1a transit peptide that was previously used for PHB production (Nawrath, C. et al. *Proc. Natl. Acad. Sci.* 91: 12760-12764, 1994), except PhbB was fused to the transit peptide from pea RuBisCo small subunit (Cashmore, A.R. Nuclear genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase. pp. 29-38 in Genetic Engineering of Plants, Kosuge, T., Meredith, C.P., Hollaender, A. (eds.). Plenum, New York, 1983). Each gene is controlled by the promoter from the fatty acid hydroxylase gene of *Lesquerella* (P-Lh; Broun, P., et al. *Plant J.* 13: 201-210, 1998), and the E9 3' region (Coruzzi, G., et al. *EMBO J.* 3: 1671-1679, 1984). P-Lh directs expression of these genes within the developing seed. Transformation of *Brassica* was performed as described by Fry et al. (*Plant Cell Rep.* 6: 321-325, 1987), and transformants were selected on glyphosate.

Isolation of *Brassica* seed leukoplasts and analysis of pyruvate dehydrogenase complex activity

Leukoplasts were isolated essentially as described by Kang and Rawsthorne (*Plant J.* 6: 795-805, 1994). Isolated leucoplasts were lysed by sonication and debris removed by centrifugation at 10,000 x g for 10 minutes. The crude extract was desalted using Pharmacia NAP-5 columns and the protein concentrations determined by the Bradford method (Bradford, M. *Anal. Biochem.* 72: 248-254, 1976). Five to 50 µL were added to assay mix which contained final concentrations of: 100 mM EPPS, pH 8.0; 5 mM $MgCl_2$; 2.4 mM coenzyme-A; 1.5 mM NAD^+; and 0.2 mM TPP (cocarboxylase). The reaction was initiated with addition of either pyruvate or 2-ketobutyrate substrates to final concentrations of 1.5 mM and 30 mM, respectively. To aid in analysis and ensure peak identities, ^{14}C labeled pyruvate and 2-ketobutyrate were spiked into both substrates. The reactions were quenched with 30 µL of 10% formic acid after 2 to 30 minutes. 100 µL of the reaction was injected onto a Beckman Ultrasphere HPLC column (5 µM, 4.6 mm x 15 cm) and eluted with 1 mL/minute gradient of solvent A (50 mM ammonium acetate buffer pH 6.0 containing 5% acetonitrile) going from 0 to 40 % solvent B (acetonitrile) in 15 minutes. The reaction was followed by monitoring absorbance of CoA-derived products at 230 and 260 nm using a photodiode array detector. Use of radioisotope flow detector allowed confirmation of both substrate and product peak identities. The percent conversion of added

substrates was used to determine the specific activities of the extracts. One unit equals one nmol product produced per minute per mg protein in extract.

Amino acid and 2-ketoacid analysis.

Amino Acid analysis was performed by Dr. Donald Willis at Ralston Analytical Laboratories, essentially as described by Willis (*J. Chromatog.* 408: 217-225, 1987).

Extraction and Gas Chromatography Analysis of Polymer from *Arabidopsis*

For isolation of polymer from *Arabidopsis*, stems and leaves were harvested and dehydrated by lyophilization for approximately 36 hours. The material was ground to a fine powder, and 100 mg of powder was treated with 10 mL Clorox bleach for 1 hour with shaking at room temperature. The extract was subjected to centrifugation at 1,600 x g for ten minutes, and the supernatant solutions was carefully removed. Ten mL 100% methanol were added, the solution was mixed by vortex, and then centrifuged again. After a second, identical, methanol extraction, the material was allowed to dry overnight. Polymer was extracted from the dried material with 1 mL of chloroform containing 3 μmol/mL methyl-benzoate standard and 1 mL of methanol/sulphuric acid (85:15, v/v). The tube was heated to 100°C for exactly 2.5 hours, and the solid material was removed by centrifugation. The solution was cooled, 1 mL water was added, and the liquid was mixed using a vortex mixer. The organic and aqueous phases were separated by centrifugation at 1,600 x g for ten minutes. The chloroform layer was transferred to a clean test tube and vigorously mixed with approximately 200 mg of silica gel. Solid material was removed by centrifugation, and the supernatant material was subjected to gas chromatography. Gas chromatographic characterization of the methyl-ester residues was performed as described by Slater et al. (*J. Bacteriol.* 180: 1979-1987, 1998), except that the temperature gradient was performed as follows. The initial temperature of 70°C was held for 6 minutes, then the temperature was increased by 30°C per minute to 130°C. Finally, the temperature was increased by 50°C per minute to 300°C and held at 300°C for 5 minutes.

Extraction and Gas Chromatography Analysis of Polymer from *Brassica* seeds

For isolation of polymer from canola seed, seeds were ground to a fine powder with a mortar and pestle. Approximately 200 mg of each sample were extracted two times in a glass tube with 10 mL each of hexane for 1 hour at 60°C, then two times with 10 mL each of 100% methanol for one hour at 60°C. This procedure removes oil from the seed. The material was allowed to dry to completion overnight. Polymer was extracted from the dried material with 1 mL of chloroform containing 3 μmol/mL methyl-benzoate standard. The tube was heated to 100°C for 5 hours and the samples were cooled. One mL methanol/sulphuric acid (85:15, v/v) was added, and the mixture was heated to 100°C for exactly 2.5 hours. The solution was cooled, extracted with water and subjected to gas chromatography as described above.

Characterization of polymer by nuclear magnetic resonance spectroscopy and gel permeation chromatography

Nuclear magnetic resonance (NMR) studies were done using a Varian Unity 500 MHz spectrometer. Proton spectra were obtained on a Varian pfg 5 mm probe at 30°C from PHA samples of approximately 20 mg dissolved in 1 mL deuterochloroform. Acquisitions were taken at a 90° pulse, 2.3 s acquisition time, 30 s delay, collecting 65k data points and 16 accumulations. Chemical shifts were referenced to $CHCl_3$ (δ=7.24 ppm). The 13C{1H} spectra (125 MHz) were taken at 30°C on a Nalorac 3 mm ^{13}C probe containing a solution of approximately 10 mg PHA in 200 μL deuterochloroform. The spectra were obtained using 30° pulses, 1.5 s acquisition time, zero delay, 131k data points and 55,296 accumulations. Chemical shifts were measured relative to $CHCl_3$ (δ=77.0 ppm).

Gel permeation chromatography was performed according to Koizumi et al. (*J. M. S. Pure Appl. Chem.* A32: 759-774, 1995).

EXAMPLE 13: Plant Promoters

Plant promoter sequences can be constitutive or inducible, environmentally- or developmentally-regulated, or cell- or tissue-specific. Often-used constitutive promoters include

the CaMV 35S promoter (Odell et al., *Nature* 313: 810-812, 1985), the enhanced CaMV 35S promoter, the Figwort Mosaic Virus (FMV) promoter (Richins, R.D. et al., *Nucleic Acids Res.* 20: 8451-8466, 1987), the mannopine synthase (*mas*) promoter, the nopaline synthase (*nos*) promoter, and the octopine synthase (*ocs*) promoter. Useful inducible promoters include promoters induced by salicylic acid or polyacrylic acids (PR-1, Williams , S. W. et al, *Biotechnology* 10: 540-543, 1992), induced by application of safeners (substituted benzenesulfonamide herbicides, Hershey, H.P. and Stoner, T.D., *Plant Mol. Biol.* 17: 679-690, 1991), heat-shock promoters (Ou-Lee et al., *Proc. Natl. Acad. Sci. U.S.A.* 83: 6815-6819, 1986; Ainley, W.M. et al., *Plant Mol. Biol.* 14: 949-967, 1990), a nitrate-inducible promoter derived from the spinach nitrite reductase gene (Back, E. et al., *Plant Mol. Biol.* 17: 9-18, 1991), hormone-inducible promoters (Yamaguchi-Shinozaki, K. et al., *Plant Mol. Biol.* 15: 905-912, 1990; Kares et al., *Plant Mol. Biol.* 15: 905-912, 1990), and light-inducible promoters associated with the small subunit of RuBP carboxylase and LHCP gene families (Kuhlemeier et al., *Plant Cell* 1: 471-478, 1989; Feinbaum, R.L. et al., *Mol. Gen. Genet.* 226: 449-456, 1991; Weisshaar, B. et al., *EMBO J.* 10: 1777-1786, 1991; Lam, E. and Chua, N.H., *J. Biol. Chem.* 266: 17131-17135, 1990; Castresana, C. et al., *EMBO J.* 7: 1929-1936, 1988; Schulze-Lefert, P. et al., *EMBO J.* 8: 651-656, 1989). Examples of useful tissue-specific, developmentally-regulated promoters include the β-conglycinin 7S promoter (Doyle, J.J. et al., *J. Biol. Chem.* 261: 9228-9238, 1986; Slighton and Beachy, *Planta* 172: 356, 1987), and seed-specific promoters (Knutzon, D.S. et al., *Proc. Natl. Acad. Sci. U.S.A.* 89: 2624-2628, 1992; Bustos, M.M. et al., *EMBO J.* 10: 1469-1479, 1991; Lam, E. and Chua, N.H., *Science* 248: 471-474, 1991; Stayton et al., *Aust. J. Plant. Physiol.* 18: 507, 1991). Plant functional promoters useful for preferential expression in seed plastids include those from plant storage protein genes and from genes involved in fatty acid biosynthesis in oilseeds. Examples of such promoters include the 5' regulatory regions from such genes as napin (Kridl et al., *Seed Sci. Res.* 1: 209-219, 1991), phaseolin, zein, soybean trypsin inhibitor, ACP, stearoyl-ACP desaturase, and oleosin. Seed-specific gene regulation is discussed in EP 0 255 378. Promoter hybrids can also be constructed to enhance transcriptional activity (Comai, L. and Moran, P.M., U.S. Patent No. 5,106,739, issued April 21, 1992), or to combine desired transcriptional activity and tissue specificity.

EXAMPLE 14: Plant transformation and regeneration

A variety of different methods can be employed to introduce such vectors into plant protoplasts, cells, callus tissue, leaf discs, meristems, etcetera, to generate transgenic plants, including *Agrobacterium*-mediated transformation, particle gun delivery, microinjection, electroporation, polyethylene glycol mediated protoplast transformation, liposome-mediated transformation, etc. (reviewed in Potrykus, *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 42: 205-225, 1991). In general, transgenic plants comprising cells containing and expressing DNAs encoding enzymes facilitating PHA biosynthesis can be produced by transforming plant cells with a DNA construct as described above via any of the foregoing methods; selecting plant cells that have been transformed on a selective medium; regenerating plant cells that have been transformed to produce differentiated plants; and selecting a transformed plant which expresses the enzyme-encoding nucleotide sequence.

Specific methods for transforming a wide variety of dicots and obtaining transgenic plants are well documented in the literature (Gasser and Fraley, *Science* 244: 1293-1299, 1989; Fisk and Dandekar, *Scientia Horticulturae* 55: 5-36, 1993; Christou, *Agro Food Industry Hi Tech*, p.17 (1994); and the references cited therein).

Successful transformation and plant regeneration have been reported in the monocots as follows: asparagus (*Asparagus officinalis*; Bytebier et al., *Proc. Natl. Acad. Sci. U.S.A.* 84: 5345-5349, 1987); barley (*Hordeum vulgarae*; Wan and Lemaux, *Plant Physiol.* 104: 37-48, 1994); maize (*Zea mays*; Rhodes, C.A. et al., Science 240: 204-207, 1988; Gordon-Kamm et al., *Plant Cell* 2: 603-618, 1990; Fromm, M.E. et al., *Bio/Technology* 8: 833-839, 1990; Koziel et al., *Bio/Technology* 11: 194-200, 1993); oats (*Avena sativa*; Somers et al., *Bio/Technology* 10: 1589-1594, 1992); orchardgrass (*Dactylis glomerata*; Horn et al., *Plant Cell Rep.* 7: 469-472, 1988); rice (*Oryza sativa*, including indica and japonica varieties; Toriyama et al., *Bio/Technology* 6: 10, 1988; Zhang et al., *Plant Cell Rep.* 7: 379-384, 1988; Luo and Wu, *Plant Mol. Biol.* Rep. 6: 165, 1988; Zhang and Wu, *Theor. Appl. Genet.* 76: 835, 1988; Christou et al., *Bio/Technology* 9: 957-962, 1991); rye (*Secale cereale*; De la Pena et al., *Nature* 325: 274-276, 1987); sorghum (*Sorghum bicolor*; Casas, A.M. et al., *Proc. Natl. Acad. Sci. U.S.A.* 90: 11212-11216, 1993); sugar cane (*Saccharum* spp.; Bower and Birch, *Plant J.* 2: 409-416, 1992); tall fescue (*Festuca arundinacea*; Wang, Z.Y. et al., *Bio/Technology* 10: 691-696, 1992); turfgrass (*Agrostis*

palustris; Zhong et al., *Plant Cell Rep.* 13: 1-6, 1993); wheat (*Triticum aestivum*; Vasil et al., *Bio/Technology* 10: 667-674, 1992; Weeks, T. et al., *Plant Physiol.* 102: 1077-1084, 1993; Becker et al., *Plant J.* 5: 299-307, 1994), and alfalfa (Masoud, S.A. et al., *Transgen. Res.* 5: 313, 1996).

EXAMPLE 15: Host plants

Particularly useful plants for polyhydroxyalkanoate production include those that produce carbon substrates which can be employed for polyhydroxyalkanoate biosynthesis, including tobacco, wheat, potato, *Arabidopsis*, and high oil seed plants such as corn, soybean, canola, oil seed rape, sunflower, flax, peanut, sugarcane, switchgrass, and alfalfa.

If the host plant of choice does not produce the requisite fatty acid substrates in sufficient quantities, it can be modified, for example by mutagenesis or genetic transformation, to block or modulate the glycerol ester and fatty acid biosynthesis or degradation pathways so that it accumulates the appropriate substrates for polyhydroxyalkanoate production. Expression of enzymes such as acyl-ACP thioesterase, fatty acyl hydroxylase, and yeast MFP may serve to increase the flux of substrates in the peroxysome, leading to higher levels of polyhydroxyalkanoate biosynthesis.

EXAMPLE 16: Nucleic acid mutation and hybridization

Variations in the nucleic acid sequence encoding a fusion protein may lead to mutant protein sequences that display equivalent or superior enzymatic characteristics when compared to the sequences disclosed herein. This invention accordingly encompasses nucleic acid sequences which are similar to the sequences disclosed herein, protein sequences which are similar to the sequences disclosed herein, and the nucleic acid sequences that encode them. Mutations may include deletions, insertions, truncations, substitutions, fusions, and the like.

Mutations to a nucleic acid sequence may be introduced in either a specific or random manner, both of which are well known to those of skill in the art of molecular biology. A myriad of site-directed mutagenesis techniques exist, typically using oligonucleotides to introduce mutations at specific locations in a nucleic acid sequence. Examples include single strand rescue

(Kunkel, T. *Proc. Natl. Acad. Sci. U.S.A.*, 82: 488-492, 1985), unique site elimination (Deng and Nickloff, *Anal. Biochem.* 200: 81, 1992), nick protection (Vandeyar, et al. *Gene* 65: 129-133, 1988), and PCR (Costa, et al. *Methods Mol. Biol.* 57: 31-44, 1996). Random or non-specific mutations may be generated by chemical agents (for a general review, see Singer and Kusmierek, *Ann. Rev. Biochem.* 52: 655-693, 1982) such as nitrosoguanidine (Cerda-Olmedo et al., *J. Mol. Biol.* 33: 705-719, 1968; Guerola, et al. *Nature New Biol.* 230: 122-125, 1971) and 2-aminopurine (Rogan and Bessman, *J. Bacteriol.* 103: 622-633, 1970), or by biological methods such as passage through mutator strains (Greener et al. *Mol. Biotechnol.* 7: 189-195, 1997).

Nucleic acid hybridization is a technique well known to those of skill in the art of DNA manipulation. The hybridization properties of a given pair of nucleic acids is an indication of their similarity or identity. Mutated nucleic acid sequences may be selected for their similarity to the disclosed nucleic acid sequences on the basis of their hybridization to the disclosed sequences. Low stringency conditions may be used to select sequences with multiple mutations. One may wish to employ conditions such as about 0.15 M to about 0.9 M sodium chloride, at temperatures ranging from about 20°C to about 55°C. High stringency conditions may be used to select for nucleic acid sequences with higher degrees of identity to the disclosed sequences. Conditions employed may include about 0.02 M to about 0.15 M sodium chloride, about 0.5% to about 5% casein, about 0.02% SDS and/or about 0.1% N-laurylsarcosine, about 0.001 M to about 0.03 M sodium citrate, at temperatures between about 50°C and about 70°C. More preferably, high stringency conditions are 0.02 M sodium chloride, 0.5% casein, 0.02% SDS, 0.001 M sodium citrate, at a temperature of 50°C.

EXAMPLE 17: Determination of homologous and degenerate nucleic acid sequences

Modification and changes may be made in the sequence of the proteins of the present invention and the nucleic acid segments which encode them and still obtain a functional molecule that encodes a protein with desirable properties. The following is a discussion based upon changing the amino acid sequence of a protein to create an equivalent, or possibly an improved, second-generation molecule. The amino acid changes may be achieved by changing the codons of the nucleic acid sequence, according to the codons given in Table 9.

Table 9: Codon degeneracies of amino acids

Amino acid	One letter	Three letter	Codons
Alanine	A	Ala	GCA GCC GCG GCT
Cysteine	C	Cys	TGC TGT
Aspartic acid	D	Asp	GAC GAT
Glutamic acid	E	Glu	GAA GAG
Phenylalanine	F	Phe	TTC TTT
Glycine	G	Gly	GGA GGC GGG GGT
Histidine	H	His	CAC CAT
Isoleucine	I	Ile	ATA ATC ATT
Lysine	K	Lys	AAA AAG
Leucine	L	Leu	TTA TTG CTA CTC CTG CTT
Methionine	M	Met	ATG
Asparagine	N	Asn	AAC AAT
Proline	P	Pro	CCA CCC CCG CCT
Glutamine	Q	Gln	CAA CAG
Arginine	R	Arg	AGA AGG CGA CGC CGG CGT
Serine	S	Ser	AGC AGT TCA TCC TCG TCT
Threonine	T	Thr	ACA ACC ACG ACT
Valine	V	Val	GTA GTC GTG GTT
Tryptophan	W	Trp	TGG
Tyrosine	Y	Tyr	TAC TAT

Certain amino acids may be substituted for other amino acids in a protein sequence without appreciable loss of enzymatic activity. It is thus contemplated that various changes may be made in the peptide sequences of the disclosed protein sequences, or their corresponding nucleic acid sequences without appreciable loss of the biological activity.

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte and Doolittle, *J. Mol. Biol.*, 157: 105-132, 1982). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics. These are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4);

threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate/glutamine/aspartate/asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, i.e., still obtain a biologically functional protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those within ±1 are more preferred, and those within ±0.5 are most preferred.

It is also understood in the art that the substitution of like amino acids may be made effectively on the basis of hydrophilicity. U.S. Patent No. 4,554,101 (Hopp, T.P., issued November 19, 1985) states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein. The following hydrophilicity values have been assigned to amino acids: arginine/lysine (+3.0); aspartate/glutamate (+3.0 ±1); serine (+0.3); asparagine/glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ±1); alanine/histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine/isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); and tryptophan (-3.4).

It is understood that an amino acid may be substituted by another amino acid having a similar hydrophilicity score and still result in a protein with similar biological activity, i.e., still obtain a biologically functional protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those within ±1 are more preferred, and those within ±0.5 are most preferred.

As outlined above, amino acid substitutions are therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine, and isoleucine. Changes which are not expected to be advantageous may also be used if these resulted in functional fusion proteins.

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the

compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the methods described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention.

TABLE 10: RESTRICTION SITES FROM FIG. 3

ENZYME	CUT SITE	ENZYME	CUT SITE
NotI	693	DraI	7754
XhoI	702	BglII	8440
BsaAI	1510	RsrII	8998
RsrII	1722	BglII	9296
XhoI	2170	AscI	9851
DraI	2817	SexAI	9917
BsaAI	4975	BsaAI	9933
DraI	5980	SfiI	10387
DraI	5999	SbfI	10535
BsaAI	7195	EcoRI	10594
DraI	7677		

TABLE 11: RESTRICTION SITES FROM FIG. 4

ENZYME	CUT SITE	ENZYME	CUT SITE
NotI	678	DraI	5929
XhoI	687	DraI	5948
BsaAI	1497	BsaAI	7144
RsrII	1709	DraI	7626
XhoI	2157	DraI	7703
DraI	2804	BglII	8389
BsaAI	4924	EcoRI	8413

TABLE 12: RESTRICTION SITES FROM FIG. 5

ENZYME	CUT SITE	ENZYME	CUT SITE
BsaAI	411	SrfI	2240
NotI	878	NotI	2244
BglII	1541	DraI	3368
EcoRI	1555	DraI	3387
SmaI	1573	DraI	4079
SmaI	2240		

TABLE 13: RESTRICTION SITES FROM FIG. 6

ENZYME	CUT SITE	ENZYME	CUT SITE
BsaAI	411	SmaI	3811
NotI	878	SrfI	3811
BglII	1541	NotI	3815
BsaAI	2185	DraI	4939
EcoRI	3094	DraI	4958
EcoRI	3126	DraI	5650
SmaI	3144		

TABLE 14: RESTRICTION SITES FROM FIG. 7

ENZYME	CUT SITE	ENZYME	CUT SITE
BsaAI	411	SmaI	3101
NotI	878	SmaI	3768
BglII	1541	SrfI	3768
BsaAI	2019	NotI	3772
SbfI	2150	DraI	4896
BsaAI	2523	DraI	4915
SbfI	2789	DraI	5607
EcoRI	3083		

TABLE 15: RESTRICTION SITES FROM FIG. 8

ENZYME	CUT SITE	ENZYME	CUT SITE
BsaAI	411	SmaI	3320
NotI	878	SrfI	3320
BglII	1541	NotI	3324
SfiI	2259	DraI	4448
EcoRI	2603	DraI	4467
EcoRI	2635	DraI	5159
SmaI	2653		

TABLE 16: RESTRICTION SITES FROM FIG. 9

ENZYME	CUT SITE	ENZYME	CUT SITE
BsaAI	411	SmaI	4029
NotI	878	SrfI	4029
BglII	1541	NotI	4033
BsaAI	3070	DraI	5157
EcoRI	3131	DraI	5176
BsaAI	3183	DraI	5868

TABLE 17: RESTRICTION SITES FROM FIG. 10

ENZYME	CUT SITE	ENZYME	CUT SITE	ENZYME	CUT SITE
NotI	693	BglII	3814	BspHI	11793
HindIII	704	HindIII	3820	SphI	12986
EcoRV	1241	HindIII	3832	HindIII	13143
BglII	1356	SphI	4136	EcoRV	13677
HindIII	1362	BspHI	4138	BglII	13792
HindIII	1374	NcoI	5005	SphI	13971
SphI	1678	EcoRI	5356	SphI	14061
SfiI	2118	SmaI	5374	NcoI	14066
NcoI	2166	BamHI	5380	EcoRV	14277
EcoRI	2462	SmaI	6041	NcoI	14321
SmaI	2480	NotI	6045	BglII	14648
BamHI	2486	XhoI	6054	SexAI	15269
SmaI	3147	SphI	6963	SfiI	15739
NotI	3151	NcoI	6990	EcoRI	15946
HindIII	3162	XhoI	7522	BamHI	15964
EcoRV	3699	BspHI	11293		

TABLE 18: RESTRICTION SITES FROM FIG. 11

ENZYME	CUT SITE	ENZYME	CUT SITE	ENZYME	CUT SITE
NotI	693	BglII	4293	BspHI	11324
HindIII	704	SphI	4472	BspHI	11824
EcoRV	1241	SphI	4562	SphI	13017
BglII	1356	NcoI	4567	HindII	13174
SphI	1535	SfiI	5011	EcoRV	13708
SphI	1625	NcoI	5059	BglII	13823
NcoI	1630	EcoRI	5355	SphI	14002
ApaI	2508	EcoRI	5387	SphI	14092
EcoRI	2909	SmaI	5405	NcoI	14097
EcoRI	2941	BamHI	5411	EcoRV	14308
SmaI	2959	SmaI	6072	NcoI	14352
BamHI	2965	NotI	6076	BglII	14679
SmaI	3626	XhoI	6085	SexAI	15300
NotI	3630	SphI	6994	SfiI	15770
HindIII	3641	NcoI	7021	EcoRI	15977
EcoRV	4178	XhoI	7553	BamHI	15995

TABLE 19: RESTRICTION SITES FROM FIG. 12

ENZYME	CUT SITE	ENZYME	CUT SITE	ENZYME	CUT SITE
NotI	693	EcoRI	5312	DraI	11339
BglII	1356	EcoRI	5344	BsaAI	12535
BsaAI	1834	SmaI	5362	DraI	13017
SbfI	1965	SmaI	6029	DraI	13094
BsaAI	2338	SrfI	6029	BglII	13780
SbfI	2604	NotI	6033	RsrII	14338
EcoRI	2898	XhoI	6042	BglII	14636
SmaI	2916	BsaAI	6850	AscI	15191
SmaI	3583	RsrII	7062	SexAI	15257
SrfI	3583	XhoI	7510	BsaAI	15273
NotI	3587	DraI	8157	SfiI	15727
BglII	4250	BsaAI	10315	SbfI	15875
SfiI	4968	DraI	11320	EcoRI	15934

TABLE 20: RESTRICTION SITES FROM FIG. 13

ENZYME	CUT SITE	ENZYME	CUT SITE	ENZYME	CUT SITE
NotI	693	BglII	4334	BspHI	11365
HindIII	704	SphI	4513	BspHI	11865
EcoRV	1241	SphI	4603	SphI	13058
BglII	1356	NcoI	4608	HindIII	13215
SphI	1535	SfiI	5052	EcoRV	13749
SphI	1625	NcoI	5100	BglII	13864
NcoI	2497	EcoRI	5396	SphI	14043
HindIII	2938	EcoRI	5428	SphI	14133
EcoRV	2946	SmaI	5446	NcoI	14138
EcoRI	2950	BamHI	5452	EcoRV	14349
EcoRI	2982	SmaI	6113	NcoI	14393
SmaI	3000	NotI	6117	BglII	14720
BamHI	3006	XhoI	6126	SexAI	15341
SmaI	3667	SphI	7035	SfiI	15811
NotI	3671	NcoI	7062	EcoRI	16018
HindIII	3682	XhoI	7594	BamHI	16036
EcoRV	4219				

TABLE 21: RESTRICTION SITES FROM FIG. 14

ENZYME	CUT SITE	ENZYME	CUT SITE	ENZYME	CUT SITE
NotI	693	NcoI	5702	XhoI	9197
HindIII	704	EcoRI	6053	SphI	10106
EcoRV	1241	SmaI	6071	NcoI	10133
BglII	1356	BamHI	6077	XhoI	10665
SphI	1535	SmaI	6738	BspHI	14436
SphI	1625	NotI	6742	BspHI	14936
NcoI	1630	HindIII	6753	SphI	16129
EcoRI	2946	EcoRV	7290	HindII	16286
SnaBI	2998	BglI	7405	EcoRV	16820
NcoI	3032	SphI	7584	BglII	16935
EcoRV	3179	SphI	7674	SphI	17114
BamHI	3183	NcoI	7679	SphI	17204
SmaI	3844	SfiI	8123	NcoI	17209
NotI	3848	NcoI	8171	EcoRV	17420
HindIII	3859	EcoRI	8467	NcoI	17464
EcoRV	4396	EcoRI	8499	BglII	17791
BglII	4511	SmaI	8517	SexAI	18412
HindIII	4517	BamHI	8523	SfiI	18882
HindIII	4529	SmaI	9184	EcoRI	19089
SphI	4833	NotI	9188	BamHI	19107
BspHI	4835				

TABLE 22: RESTRICTION SITES FROM FIG. 15

ENZYME	CUT SITE	ENZYME	CUT SITE	ENZYME	CUT SITE
NotI	693	SmaI	6999	SmaI	11672
HindIII	704	NotI	7003	BamHI	11678
EcoRV	1241	HindIII	7014	SmaI	12339
BglII	1356	EcoRV	7551	NotI	12343
SphI	1535	BglII	7666	XhoI	12352
SphI	1625	HindIII	7672	SphI	13261
NcoI	1630	HindIII	7684	NcoI	13288
EcoRI	2946	SphI	7988	XhoI	13820
SnaBI	2998	BspHI	7990	BspHI	17591
NcoI	3032	NcoI	8857	BspHI	18091
EcoRV	3179	EcoRI	9208	SphI	19284
BamHI	3183	SmaI	9226	HindIII	19441
SmaI	3844	BamHI	9232	EcoRV	19975
NotI	3848	SmaI	9893	BglII	20090
HindIII	3859	NotI	9897	SphI	20269
EcoRV	4396	HindIII	9908	SphI	20359
BglII	4511	EcoRV	10445	NcoI	20364
SphI	4690	BglII	10560	EcoRV	20575
SphI	4780	SphI	10739	NcoI	20619
NcoI	4785	SphI	10829	BglII	20946
EcoRI	6101	NcoI	10834	SexAI	21567
SnaBI	6153	SfiI	11278	SfiI	22037
NcoI	6187	NcoI	11326	EcoRI	22244
EcoRV	6334	EcoRI	11622	BamHI	22262
BamHI	6338	EcoRI	11654		

TABLE 23: RESTRICTION SITES FROM FIG. 16

ENZYME	CUT SITE	ENZYME	CUT SITE	ENZYME	CUT SITE
NotI	678	DraI	774	BsaAI	8144
SpeI	685	SwaI	774	BsaAI	8164
BsaAI	693	XhoI	779	DraI	8394
SanDI	698	DraI	1426	BglII	8582
RsrII	705	BsaAI	3546	RsrII	9140
SexAI	711	DraI	4551	BglII	9438
PacI	722	DraI	4570	AscI	9993
SgfI	730	BsaAI	5766	SexAI	10059
SfiI	741	DraI	6248	BsaAI	10075
AscI	748	DraI	6325	SfiI	10529
SbfI	760	DraI	6424	SbfI	10677
SmaI	766	PacI	7426	EcoRI	10736
SrfI	766	DraI	7887		

TABLE 24: RESTRICTION SITES FROM FIG. 17

ENZYME	CUT SITE	ENZYME	CUT SITE	ENZYME	CUT SITE
NotI	678	SmaI	766	DraI	6248
SpeI	685	SrfI	766	DraI	6325
BsaAI	693	DraI	774	DraI	6424
SanDI	698	SwaI	774	PacI	7426
RsrII	705	XhoI	779	DraI	7887
SexAI	711	DraI	1426	BsaAI	8144
PacI	722	BsaAI	3546	BsaAI	8164
SgfI	730	DraI	4551	DraI	8394
SfiI	741	DraI	4570	BglII	8582
AscI	748	BsaAI	5766	EcoRI	8606
SbfI	760				

TABLE 25: RESTRICTION SITES FROM FIG. 18

ENZYME	CUT SITE	ENZYME	CUT SITE
BsaAI	411	EcoRI	3123
NotI	878	SmaI	3141
DraI	951	SmaI	3808
PacI	1953	SrfI	3808
DraI	2414	NotI	3812
BsaAI	2671	DraI	4936
BsaAI	2691	DraI	4955
DraI	2921	DraI	5647
BglII	3109		

TABLE 26: RESTRICTION SITES FROM FIG. 19

ENZYME	CUT SITE	ENZYME	CUT SITE
BsaAI	411	BsaAI	3753
NotI	878	EcoRI	4662
DraI	951	SmaI	4680
PacI	1953	SmaI	5347
DraI	2414	SrfI	5347
BsaAI	2671	NotI	5351
BsaAI	2691	DraI	6475
DraI	2921	DraI	6494
BglII	3109	DraI	7186

TABLE 27: RESTRICTION SITES FROM FIG. 20

ENZYME	CUT SITE	ENZYME	CUT SITE
NotI	878	HindIII	4691
HindIII	889	EcoRV	4699
SphI	1041	EcoRI	4703
PacI	1953	SmaI	4721
BspHI	2613	BamHI	4727
BspHI	2736	SmaI	5388
BglII	3109	NotI	5392
SphI	3288	BspHI	6477
SphI	3378	BspHI	7485
NcoI	4250	BspHI	7590

TABLE 28: RESTRICTION SITES FROM FIG. 21

ENZYME	CUT SITE	ENZYME	CUT SITE	ENZYME	CUT SITE
XhoI	271	DraI	445	SanDI	4317
DraI	280	PacI	1447	RsrII	4324
SwaI	280	DraI	1908	SexAI	4330
SmaI	288	BsaAI	2165	PacI	4341
SrfI	288	BsaAI	2185	SgfI	4349
SbfI	298	DraI	2415	SfiI	4361
AscI	302	SpeI	2609	AscI	4368
SfiI	316	SmaI	2867	SbfI	4380
SgfI	326	SfiI	3315	SmaI	4386
PacI	334	EcoRI	3608	SrfI	4386
SexAI	338	SmaI	3626	DraI	4394
RsrII	346	SmaI	4293	SwaI	4394
SanDI	353	SrfI	4293	DraI	5388
BsaAI	361	NotI	4297	DraI	5407
SpeI	365	SpeI	4304	DraI	6099
NotI	372	BsaAI	4312		

TABLE 29: RESTRICTION SITES FROM FIG. 22

ENZYME	CUT SITE	ENZYME	CUT SITE
BsaAI	411	BsaAI	4638
NotI	878	EcoRI	4699
DraI	951	BsaAI	4751
PacI	1953	SmaI	5597
DraI	2414	SrfI	5597
BsaAI	2671	NotI	5601
BsaAI	2691	DraI	6725
DraI	2921	DraI	6744
BglII	3109	DraI	7436

TABLE 30: RESTRICTION SITES FROM FIG. 23

ENZYME	CUT SITE	ENZYME	CUT SITE	ENZYME	CUT SITE
NotI	678	SpeI	7388	NotI	11596
HindIII	689	EcoRV	7413	BspHI	15339
SphI	841	SphI	7573	BspHI	15839
PacI	1753	SmaI	7646	SphI	17032
BspHI	2413	SfiI	8094	HindIII	17189
BspHI	2536	NcoI	8142	SphI	17341
BglII	2909	EcoRI	8387	PacI	18253
SphI	3088	SmaI	8405	BspHI	18913
SphI	3178	BamHI	8411	BspHI	19036
NcoI	3183	BamHI	9358	BglII	19409
ApaI	4061	EcoRI	9376	SphI	19588
EcoRI	4462	BspHI	10162	SphI	19678
SmaI	4480	NcoI	10435	NcoI	19683
BamHI	4486	BamHI	10546	EcoRV	19894
SmaI	5147	NcoI	10558	NcoI	19938
NotI	5151	SfiI	10569	BglII	20265
HindIII	5162	SphI	10757	SexAI	20886
SphI	5314	BglII	10980	SfiI	21356
PacI	6226	EcoRI	11052	EcoRI	21563
BspHI	6886	EcoRI	11455	BamHI	21581
BspHI	7009	HindIII	11585		

TABLE 31: RESTRICTION SITES FROM FIG. 24

ENZYME	CUT SITE	ENZYME	CUT SITE	ENZYME	CUT SITE
NotI	678	BspHI	7050	HindIII	11626
HindIII	689	SpeI	7429	NotI	11637
SphI	841	EcoRV	7454	BspHI	15380
PacI	1753	SphI	7614	BspHI	15880
BspHI	2413	SmaI	7687	SphI	17073
BspHI	2536	SfiI	8135	HindIII	17230
BglII	2909	NcoI	8183	SphI	17382
SphI	3088	EcoRI	8428	PacI	18294
SphI	3178	SmaI	8446	BspHI	18954
NcoI	4050	BamHI	8452	BspHI	19077
HindIII	4491	BamHI	9399	BglII	19450
EcoRV	4499	EcoRI	9417	SphI	19629
EcoRI	4503	BspHI	10203	SphI	19719
SmaI	4521	NcoI	10476	NcoI	19724
BamHI	4527	BamHI	10587	EcoRV	19935
SmaI	5188	NcoI	10599	NcoI	19979
NotI	5192	SfiI	10610	BglII	20306
HindIII	5203	SphI	10798	SexAI	20927
SphI	5355	BglII	11021	SfiI	21397
PacI	6267	EcoRI	11093	EcoRI	21604
BspHI	6927	EcoRI	11496	BamHI	21622

TABLE 32: RESTRICTION SITES FROM FIG. 25

ENZYME	CUT SITE	ENZYME	CUT SITE	ENZYME	CUT SITE
NotI	678	EcoRI	9013	SphI	15521
HindIII	689	EcoRV	9246	BglII	15744
SphI	841	BamHI	9250	EcoRI	15816
PacI	1753	SmaI	9911	EcoRI	16219
BspHI	2413	NotI	9915	HindIII	16349
BspHI	2536	HindIII	9926	NotI	16360
BglII	2909	SphI	10078	BspHI	20103
SphI	3088	PacI	10990	BspHI	20603
SphI	3178	BspHI	11650	SphI	21796
NcoI	4050	BspHI	11773	HindIII	21953
HindIII	4491	SpeI	12152	SphI	22105
EcoRV	4499	EcoRV	12177	PacI	23017
EcoRI	4503	SphI	12337	BspHI	23677
SmaI	4521	SmaI	12410	BspHI	23800
BamHI	4527	SfiI	12858	BglII	24173
SmaI	5188	NcoI	12906	SphI	24352
NotI	5192	EcoRI	13151	SphI	24442
HindIII	5203	SmaI	13169	NcoI	24447
SphI	5355	BamHI	13175	EcoRV	24658
PacI	6267	BamHI	14122	NcoI	24702
BspHI	6927	EcoRI	14140	BglII	25029
BspHI	7050	BspHI	14926	SexAI	25650
BglII	7423	NcoI	15199	SfiI	26120
SphI	7602	BamHI	15310	EcoRI	26327
SphI	7692	NcoI	15322	BamHI	26345
NcoI	7697	SfiI	15333		

TABLE 33: RESTRICTION SITES FROM FIG. 26

ENZYME	CUT SITE	ENZYME	CUT SITE	ENZYME	CUT SITE
BglII	649	EcoRI	6440	DraI	8098
DraI	1202	SmaI	6712	BsaAI	8190
DraI	1278	NotI	6717	BsaAI	8731
BsaAI	1370	SpeI	6724	RsrII	8943
SfiI	2185	BsaAI	6732	EcoRI	9280
EcoRI	2529	SanDI	6737	DraI	10201
SmaI	2801	RsrII	6744	BsaAI	12321
NotI	2806	SexAI	6750	DraI	13326
BglII	3468	PacI	6761	DraI	13345
DraI	4021	SgfI	6769	BsaAI	14541
DraI	4097	SfiI	6780	DraI	15023
BsaAI	4189	AscI	6787	DraI	15100
RsrII	4844	SbfI	6799	BglII	15786
BglII	5142	SmaI	6805	DraI	16339
AscI	5697	SrfI	6805	DraI	16415
SexAI	5763	DraI	6813	BsaAI	16507
BsaAI	5779	SwaI	6813	BsaAI	17248
SfiI	6233	BglII	7469	EcoRI	18157
SbfI	6381	DraI	8022		

TABLE 34: RESTRICTION SITES FROM FIG. 27

ENZYME	CUT SITE	ENZYME	CUT SITE
EcoRV	637	EcoRV	15257
BglII	752	NotI	15268
EcoRV	2829	BglII	16310
HindIII	8420	EcoRV	17613
BglII	9445	BglII	17984
EcoRV	12082	EcoRV	19548
HindIII	12086	NotI	19559
BglIII	13111		

TABLE 35: RESTRICTION SITES FROM FIG. 28

ENZYME	CUT SITE	ENZYME	CUT SITE
EcoRV	637	EcoRV	14937
EcoRV	2829	NotI	14948
HindIII	8420	EcoRV	17133
EcoRV	11922	EcoRV	19068
HindIII	11926	NotI	19079

TABLE 36: RESTRICTION SITES FROM FIG. 29

ENZYME	CUT SITE	ENZYME	CUT SITE	ENZYME	CUT SITE
NotI	678	AscI	748	BsaAI	3546
SpeI	685	SbfI	760	DraI	4551
BsaAI	693	SmaI	766	DraI	4570
SanDI	698	SrfI	766	BsaAI	5766
RsrII	705	DraI	774	DraI	6248
SexAI	711	SwaI	774	DraI	6325
PacI	722	XhoI	779	BglII	7011
SgfI	730	DraI	1426	EcoRI	7035
SfiI	741				

TABLE 37: RESTRICTION SITES FROM FIG. 30

ENZYME	CUT SITE	ENZYME	CUT SITE	ENZYME	CUT SITE
XhoI	271	BsaAI	361	SexAI	1771
DraI	280	SpeI	365	PacI	1782
SwaI	280	NotI	372	SgfI	1790
SmaI	288	SmaI	380	SfiI	1802
SrfI	288	SrfI	380	AscI	1809
SbfI	298	SmaI	1047	SbfI	1821
AscI	302	EcoRI	1061	SmaI	1827
SfiI	316	BglII	1075	SrfI	1827
SgfI	326	NotI	1738	DraI	1835
PacI	334	SpeI	1745	SwaI	1835
SexAI	338	BsaAI	1753	DraI	2829
RsrII	346	SanDI	1758	DraI	2848
SanDI	353	RsrII	1765	DraI	3540

CLAIMS:

1. An isolated nucleic acid segment comprising:
a first nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein;
a second nucleic acid sequence encoding a β-ketoacyl reductase protein; and
a third nucleic acid sequence encoding a β-ketothiolase protein.

2. The isolated nucleic acid segment of claim 1, further comprising a fourth nucleic acid sequence encoding a threonine deaminase protein.

3. The isolated nucleic acid segment of claim 1, further comprising a fourth nucleic acid sequence encoding a deregulated threonine deaminase protein.

4. The isolated nucleic acid segment of claim 1, wherein:
the first nucleic acid sequence further encodes a chloroplast transit peptide;
the second nucleic acid sequence further encodes a chloroplast transit peptide; and
the third nucleic acid sequence further encodes a chloroplast transit peptide.

5. A recombinant vector comprising operatively linked in the 5' to 3' direction:
a promoter that directs transcription of a first nucleic acid sequence, a second nucleic acid sequence, and a third nucleic acid sequence;
a first nucleic acid sequence;
a second nucleic acid sequence;
a third nucleic acid sequence;
a 3' transcription terminator; and
a 3' polyadenylation signal sequence;
wherein:
the first nucleic acid sequence, second nucleic acid sequence, and third nucleic acid sequence encode different proteins; and
the first nucleic acid sequence, second nucleic acid sequence, and third nucleic acid sequence are independently selected from the group consisting of a nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein, a nucleic acid sequence encoding a β-ketoacyl reductase protein, and a nucleic acid sequence encoding a β-ketothiolase protein.

6. The recombinant vector of claim 5, wherein the promoter directs transcription of the first nucleic acid sequence, the second nucleic acid sequence, and the third nucleic acid sequence in plants.

7. The recombinant vector of claim 5, wherein the promoter is a viral promoter.

8. The recombinant vector of claim 5, wherein the promoter is a CMV 35S promoter, an enhanced CMV 35S promoter, or an FMV 35S promoter.

9. The recombinant vector of claim 5, wherein the promoter is an enhanced CMV 35S promoter.

10. The recombinant vector of claim 5, wherein the promoter is a tissue specific promoter.

11. The recombinant vector of claim 5, wherein the promoter is a *Lesquerella* hydroxylase promoter or a 7S conglycinin promoter.

12. The recombinant vector of claim 5, wherein the promoter is a *Lesquerella* hydroxylase promoter.

13. The recombinant vector of claim 5, wherein:
the first nucleic acid sequence further encodes a chloroplast transit peptide;
the second nucleic acid sequence further encodes a chloroplast transit peptide; and
the third nucleic acid sequence further encodes a chloroplast transit peptide.

14. A recombinant vector comprising:
a first element comprising operatively linked in the 5' to 3' direction:
a first promoter that directs transcription of a first nucleic acid sequence;
a first nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein;
a first 3' transcription terminator; and
a first 3' polyadenylation signal sequence;
a second element comprising operatively linked in the 5' to 3' direction:
a second promoter that directs transcription of a second nucleic acid sequence;
a second nucleic acid sequence encoding a β-ketoacyl reductase protein;
a second 3' transcription terminator; and
a second 3' polyadenylation signal sequence; and
a third element comprising operatively linked in the 5' to 3' direction:
a third promoter that directs transcription of a third nucleic acid sequence;
a third nucleic acid sequence encoding a β-ketothiolase protein;
a third 3' transcription terminator; and
a third 3' polyadenylation signal sequence.

15. The recombinant vector of claim 14, wherein the β-ketothiolase protein:
catalyzes the condensation of two molecules of acetyl-CoA to produce acetoacetyl-CoA; and
catalyzes the condensation of acetyl-CoA and propionyl-CoA to produce β-ketovaleryl-CoA.

16. The recombinant vector of claim 14, wherein the β-ketoacyl reductase protein:
catalyzes the reduction of acetoacetyl-CoA to β-hydroxybutyryl-CoA; and
catalyzes the reduction of β-ketovaleryl-CoA to β-hydroxyvaleryl-CoA.

17. The recombinant vector of claim 14, wherein the polyhydroxyalkanoate synthase protein is selected from the group consisting of:
a polyhydroxyalkanoate synthase protein that catalyzes the incorporation of β-hydroxybutyryl-CoA into P(3HB) polymer; and

a polyhydroxyalkanoate synthase protein that catalyzes the incorporation of β-hydroxybutyryl-CoA and β-hydroxyvaleryl-CoA into P(3HB-co-3HV) copolymer.

18. The recombinant vector of claim 14, wherein:
the β-ketothiolase protein comprises a transit peptide sequence that directs transport of the β-ketothiolase protein to the plastid;
the β-ketoacyl reductase protein comprises a transit peptide sequence that directs transport of the β-ketoacyl reductase protein to the plastid; and
the polyhydroxyalkanoate synthase protein comprises a transit peptide sequence that directs transport of the polyhydroxyalkanoate synthase protein to the plastid.

19. The recombinant vector of claim 14, further comprising a nucleic acid sequence encoding a threonine deaminase protein.

20. The recombinant vector of claim 14, further comprising a nucleic acid sequence encoding a deregulated threonine deaminase protein.

21. The recombinant vector of claim 14, wherein:
the first promoter directs transcription of the first nucleic acid sequence in plants;
the second promoter directs transcription of the second nucleic acid sequence in plants; and
the third promoter directs transcription of the third nucleic acid sequence in plants.

22. The recombinant vector of claim 14, wherein the first promoter, second promoter, and third promoter are viral promoters.

23. The recombinant vector of claim 14, wherein:
the first promoter is a CMV 35S promoter, an enhanced CMV 35S promoter, or an FMV 35S promoter;
the second promoter is a CMV 35S promoter, an enhanced CMV 35S promoter, or an FMV 35S promoter; and
the third promoter is a CMV 35S promoter, an enhanced CMV 35S promoter, or an FMV 35S promoter.

24. The recombinant vector of claim 14, wherein:
the first promoter is an enhanced CMV 35S promoter;
the second promoter is an enhanced CMV 35S promoter; and
the third promoter is an enhanced CMV 35S promoter.

25. The recombinant vector of claim 14, wherein:
the first promoter is a tissue specific promoter;
the second promoter is a tissue specific promoter; and
the third promoter is a tissue specific promoter.

26. The recombinant vector of claim 14, wherein:

the first promoter is a *Lesquerella* hydroxylase promoter or a 7S conglycinin promoter;
the second promoter is a *Lesquerella* hydroxylase promoter or a 7S conglycinin promoter; and
the third promoter is a *Lesquerella* hydroxylase promoter or a 7S conglycinin promoter.

27. The recombinant vector of claim 14, wherein:
the first promoter is a *Lesquerella* hydroxylase promoter;
the second promoter is a *Lesquerella* hydroxylase promoter; and
the third promoter is a *Lesquerella* hydroxylase promoter.

28. The recombinant vector of claim 14, wherein:
the first nucleic acid sequence further encodes a chloroplast transit peptide;
the second nucleic acid sequence further encodes a chloroplast transit peptide; and
the third nucleic acid sequence further encodes a chloroplast transit peptide.

29. A transformed host cell comprising a recombinant vector, wherein the recombinant vector comprises:
a first element comprising operatively linked in the 5' to 3' direction:
a first promoter that directs transcription of a first nucleic acid sequence;
a first nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein;
a first 3' transcription terminator; and
a first 3' polyadenylation signal sequence;
a second element comprising operatively linked in the 5' to 3' direction:
a second promoter that directs transcription of a second nucleic acid sequence;
a second nucleic acid sequence encoding a β-ketoacyl reductase protein;
a second 3' transcription terminator; and
a second 3' polyadenylation signal sequence; and
a third element comprising operatively linked in the 5' to 3' direction:
a third promoter that directs transcription of a third nucleic acid sequence;
a third nucleic acid sequence encoding a β-ketothiolase protein;
a third 3' transcription terminator; and
a third 3' polyadenylation signal sequence.

30. The transformed host cell of claim 29, wherein the transformed host cell is a bacterial cell.

31. The transformed host cell of claim 29, wherein the transformed host cell is a fungal cell.

32. The transformed host cell of claim 29, wherein the transformed host cell is a plant cell.

33. The transformed host cell of claim 29, wherein:
the first nucleic acid sequence further encodes a chloroplast transit peptide;
the second nucleic acid sequence further encodes a chloroplast transit peptide; and
the third nucleic acid sequence further encodes a chloroplast transit peptide.

34. A transformed host cell comprising:
a first element comprising operatively linked in the 5' to 3' direction:
a first promoter that directs transcription of a first nucleic acid sequence;
a first nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein;
a first 3' transcription terminator; and
a first 3' polyadenylation signal sequence;
a second element comprising operatively linked in the 5' to 3' direction:
a second promoter that directs transcription of a second nucleic acid sequence;
a second nucleic acid sequence encoding a β-ketoacyl reductase protein;
a second 3' transcription terminator; and
a second 3' polyadenylation signal sequence; and
a third element comprising operatively linked in the 5' to 3' direction:
a third promoter that directs transcription of a third nucleic acid sequence;
a third nucleic acid sequence encoding a β-ketothiolase protein;
a third 3' transcription terminator; and
a third 3' polyadenylation signal sequence;
wherein the first element, second element, and third element are cointegrated between a single left Ti border sequence and a single right Ti border sequence.

35. The transformed host cell of claim 34, wherein the transformed host cell is a fungal cell.

36. The transformed host cell of claim 34, wherein the transformed host cell is a plant cell.

37. The transformed host cell of claim 34, wherein the transformed host cell is a tobacco, wheat, potato, *Arabidopsis*, corn, soybean, canola, oil seed rape, sunflower, flax, peanut, sugarcane, switchgrass, or alfalfa cell.

38. The transformed host cell of claim 34, wherein:
the first nucleic acid sequence further encodes a chloroplast transit peptide;
the second nucleic acid sequence further encodes a chloroplast transit peptide; and
the third nucleic acid sequence further encodes a chloroplast transit peptide.

39. A transformed plant comprising:
a first element comprising operatively linked in the 5' to 3' direction:
a first promoter that directs transcription of a first nucleic acid sequence;
a first nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein;
a first 3' transcription terminator;and
a first 3' polyadenylation signal sequence;
a second element comprising operatively linked in the 5' to 3' direction:
a second promoter that directs transcription of a second nucleic acid sequence;
a second nucleic acid sequence encoding a β-ketoacyl reductase protein;
a second 3' transcription terminator; and
a second 3' polyadenylation signal sequence; and
a third element comprising operatively linked in the 5' to 3' direction:
a third promoter that directs transcription of a third nucleic acid sequence;
a third nucleic acid sequence encoding a β-ketothiolase protein;
a third 3' transcription terminator; and
a third 3' polyadenylation signal sequence;
wherein the first element, second element, and third element are cointegrated between a single left Ti border sequence and a single right Ti border sequence.

40. The transformed plant of claim 39, wherein the transformed plant is a tobacco, wheat, potato, *Arabidopsis*, corn, soybean, canola, oil seed rape, sunflower, flax, peanut, sugarcane, switchgrass, or alfalfa plant.

41. The transformed plant of claim 39, wherein:
the first nucleic acid sequence further encodes a chloroplast transit peptide;
the second nucleic acid sequence further encodes a chloroplast transit peptide; and
the third nucleic acid sequence further encodes a chloroplast transit peptide.

42. A method of preparing transformed host cells, the method comprising:
selecting a host cell;
transforming the selected host cell with a recombinant vector comprising:
a first element comprising operatively linked in the 5' to 3' direction:
a first promoter that directs transcription of the first nucleic acid sequence;
a first nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein;
a first 3' transcription terminator; and
a first 3' polyadenylation signal sequence;
a second element comprising operatively linked in the 5' to 3' direction:
a second promoter that directs transcription of the second nucleic acid sequence;
a second nucleic acid sequence encoding a β-ketoacyl reductase protein;
a second 3' transcription terminator; and
a second 3' polyadenylation signal sequence; and
a third element comprising operatively linked in the 5' to 3' direction:
a third promoter that directs transcription of the third nucleic acid sequence;
a third nucleic acid sequence encoding a β-ketothiolase protein;
a third 3' transcription terminator; and
a third 3' polyadenylation signal sequence; and
obtaining transformed host cells; wherein the transformed host cells produce polyhydroxyalkanoate polymer.

43. A method of preparing transformed host cells, the method comprising:
selecting a host cell;
transforming the selected host cell with a recombinant vector comprising operatively linked in the 5' to 3' direction:
a promoter that directs transcription of a first nucleic acid sequence, second nucleic acid sequence, and third nucleic acid sequence;
a first nucleic acid sequence;
a second nucleic acid sequence;
a third nucleic acid sequence;
a 3' transcription terminator; and
a 3' polyadenylation signal sequence; and
obtaining transformed host cells; wherein:
the first nucleic acid sequence, second nucleic acid sequence, and third nucleic acid sequence encode different proteins;
the first nucleic acid sequence, second nucleic acid sequence, and third nucleic acid sequence are independently selected from the group consisting of a nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein, a nucleic acid sequence encoding a β-ketoacyl reductase protein, and a nucleic acid sequence encoding a β-ketothiolase protein; and
the transformed host cells produce polyhydroxyalkanoate polymer.

44. A method of preparing transformed plants, the method comprising:

selecting a host plant cell;
transforming the selected host plant cell with a recombinant vector comprising:
a first element comprising operatively linked in the 5' to 3' direction:
a first promoter that directs transcription of a first nucleic acid sequence;
a first nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein;
a first 3' transcription terminator; and
a first 3' polyadenylation signal sequence;
a second element comprising operatively linked in the 5' to 3' direction:
a second promoter that directs transcription of a second nucleic acid sequence;
a second nucleic acid sequence encoding a β-ketoacyl reductase protein;
a second 3' transcription terminator; and
a second 3' polyadenylation signal sequence; and
a third element comprising operatively linked in the 5' to 3' direction:
a third promoter that directs transcription of a third nucleic acid sequence;
a third nucleic acid sequence encoding a β-ketothiolase protein;
a third 3' transcription terminator; and
a third 3' polyadenylation signal sequence;
obtaining transformed host plant cells; and
regenerating the transformed host plant cells to produce transformed plants, wherein the transformed plants produce polyhydroxyalkanoate polymer.

45. A method of preparing transformed plants, the method comprising:
selecting a host plant cell;
transforming the selected host plant cell with a recombinant vector comprising operatively linked in the 5' to 3' direction:
a promoter that directs transcription of a first nucleic acid sequence, second nucleic acid sequence, and third nucleic acid sequence;
a first nucleic acid sequence;
a second nucleic acid sequence;
a third nucleic acid sequence;
a 3' transcription terminator; and
a 3' polyadenylation signal sequence;
obtaining transformed host plant cells; and
regenerating the transformed host plant cells to produce transformed plants; wherein:
the first nucleic acid sequence, second nucleic acid sequence, and third nucleic acid sequence encode different proteins;
the first nucleic acid sequence, second nucleic acid sequence, and third nucleic acid sequence are independently selected from the group consisting of a nucleic acid sequence encoding a polyhydroxyalkanoate synthase protein, a nucleic acid sequence encoding a β-ketoacyl reductase protein, and a nucleic acid sequence encoding a β-ketothiolase protein; and
the transformed plants produce polyhydroxyalkanoate polymer.

46. A method of producing polyhydroxyalkanoate comprising:

obtaining the transformed host cell of claim 29 or claim 34;
culturing the transformed host cell under conditions suitable for the production of polyhydroxyalkanoate; and
recovering polyhydroxyalkanoate from the transformed host cell.

47. The method of claim 46, wherein the polyhydroxyalkanoate is poly(3-hydroxybutyrate), poly(4-hydroxybutyrate), or poly(3-hydroxybutyrate-co-4-hydroxybutyrate).

48. A method of producing polyhydroxyalkanoate comprising:
obtaining the transformed plant of claim 39;
growing the transformed plant under conditions suitable for the production of polyhydroxyalkanoate; and
recovering polyhydroxyalkanoate from the transformed plant.

49. The method of claim 48, wherein the polyhydroxyalkanoate is poly(3-hydroxybutyrate), poly(3-hydroxyvalerate), or poly(3-hydroxybutyrate-co-3-hydroxyvalerate).

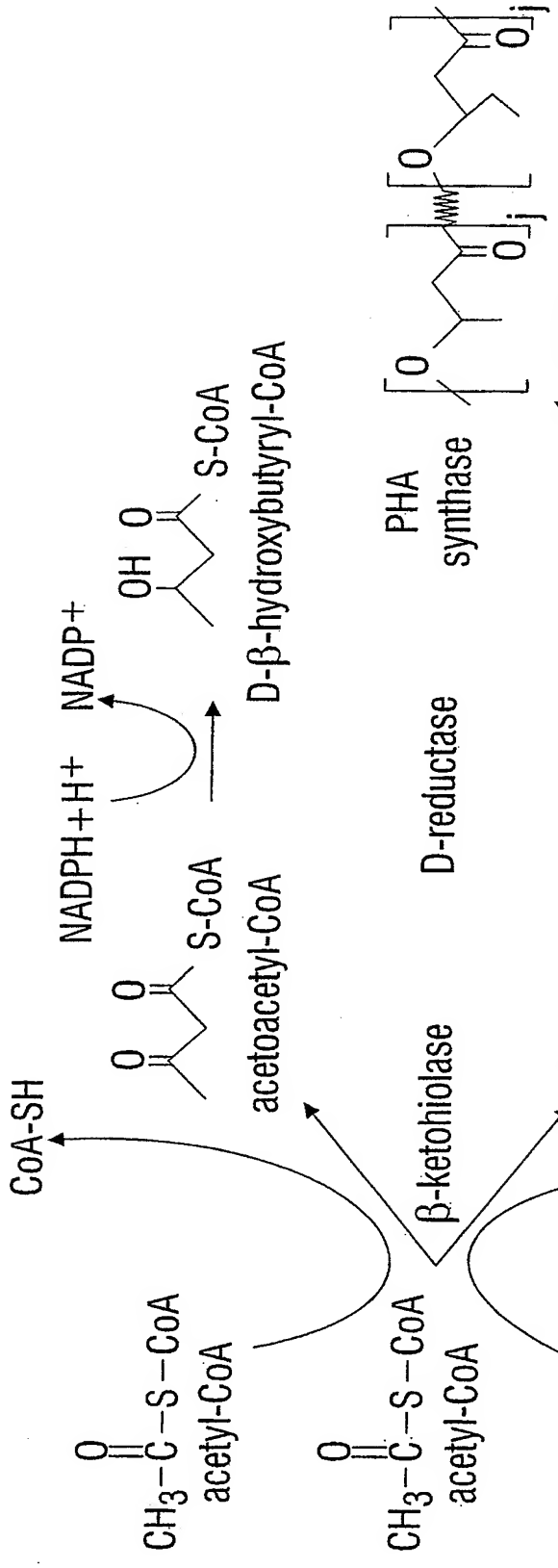
CoA-SH
acetyl-CoA
β-ketothiolase
acetoacetyl-CoA
S-CoA
NADPH+H+
NADP+
D-reductase
D-β-hydroxybutyryl-CoA
PHA synthase
PHBV copolymer
β-ketoaveryl-CoA
D-β-hydroxyvaleryl-CoA
propionyl-CoA

FIG. 1

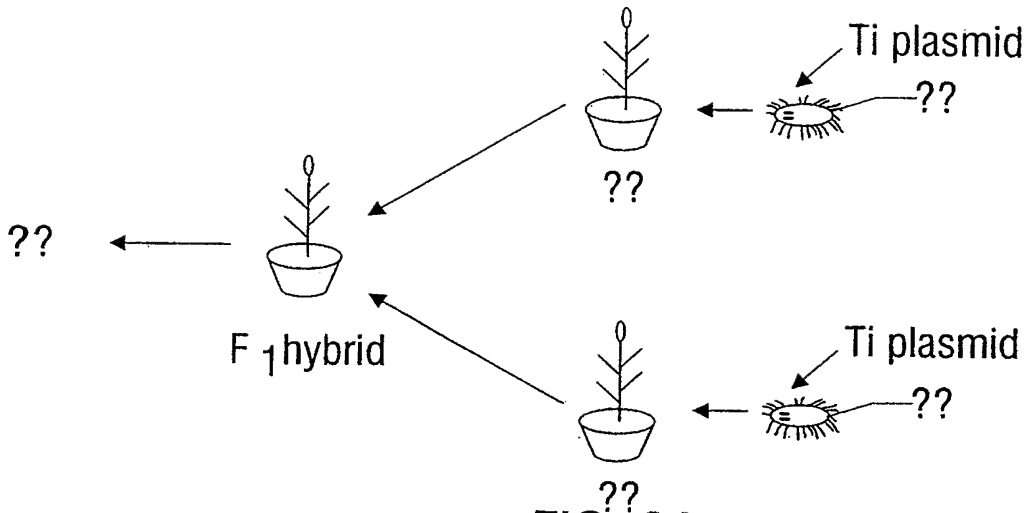

FIG. 2A

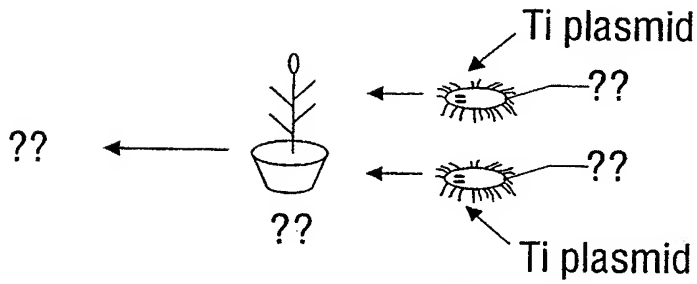

FIG. 2B

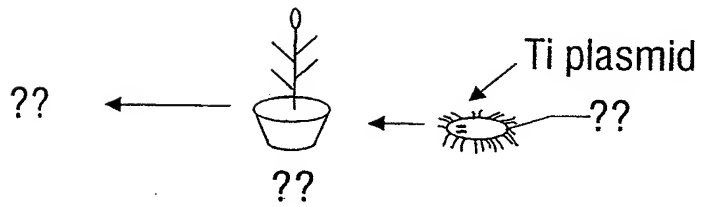

FIG. 2C

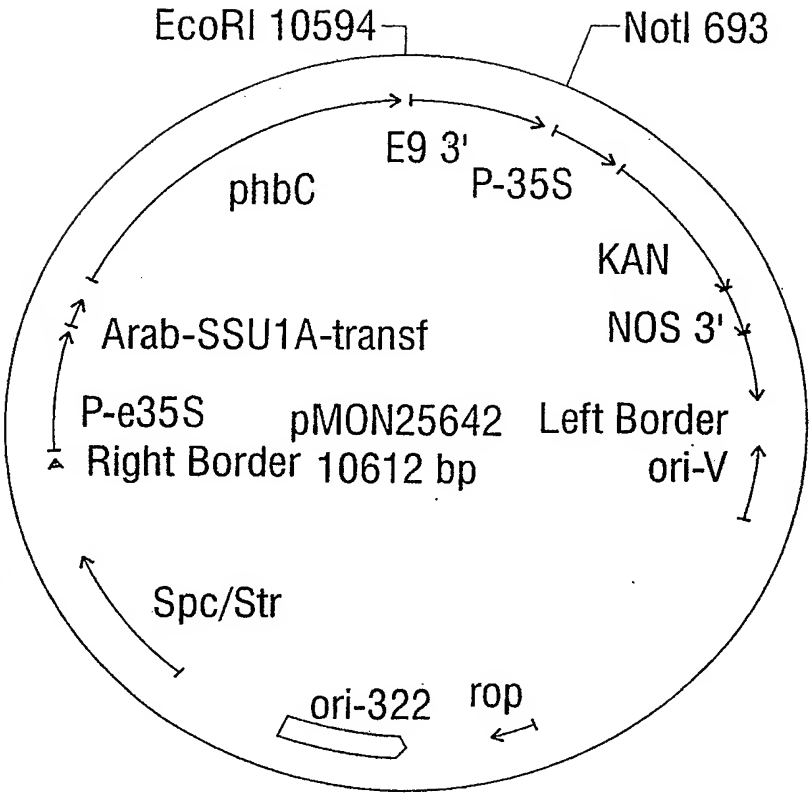
EcoRI 10594
NotI 693
E9 3'
phbC
P-35S
KAN
Arab-SSU1A-transf
NOS 3'
P-e35S
pMON25642
Left Border
Right Border
10612 bp
ori-V
Spc/Str
ori-322
rop

FIG. 3

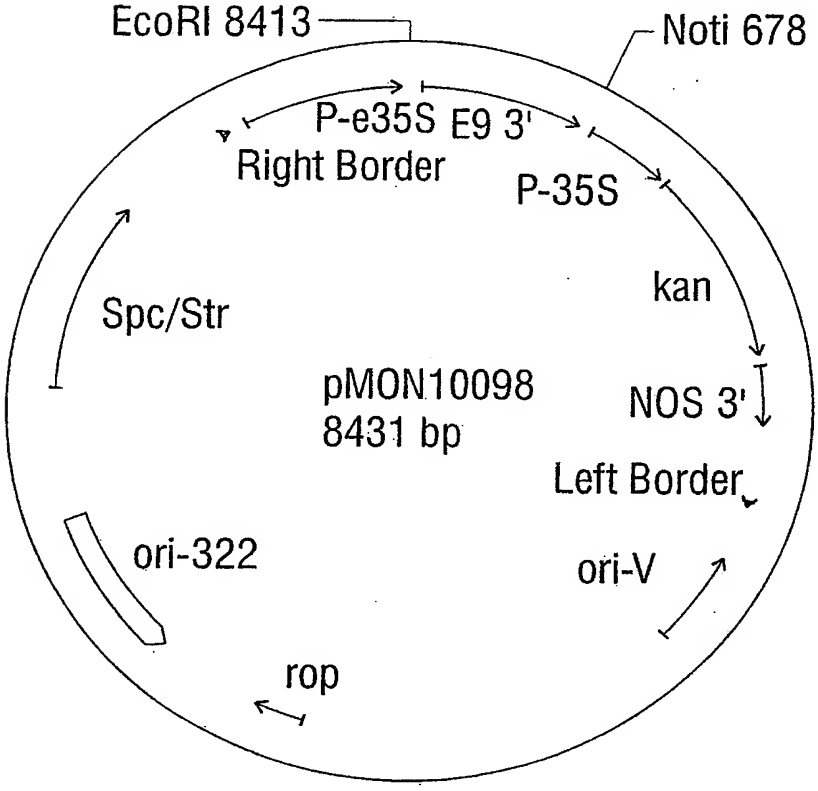
EcoRI 8413
Noti 678
P-e35S
E9 3'
Right Border
P-35S
kan
Spc/Str
pMON10098
8431 bp
NOS 3'
Left Border
ori-322
ori-V
rop

FIG. 4

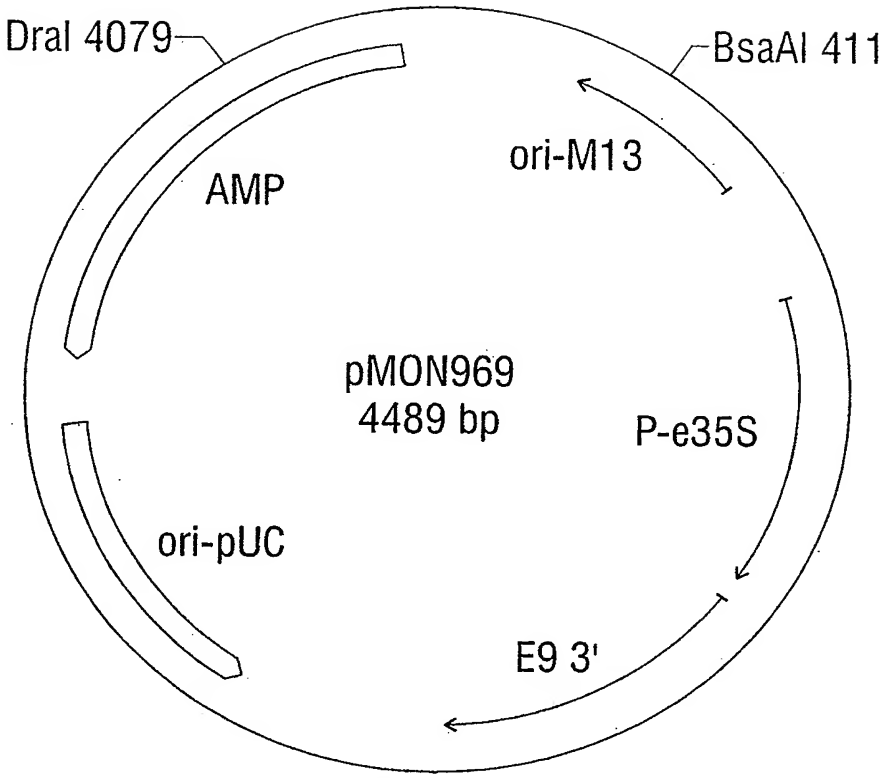
DraI 4079
BsaAI 411
ori-M13
AMP
pMON969
4489 bp
P-e35S
ori-pUC
E9 3'

FIG. 5

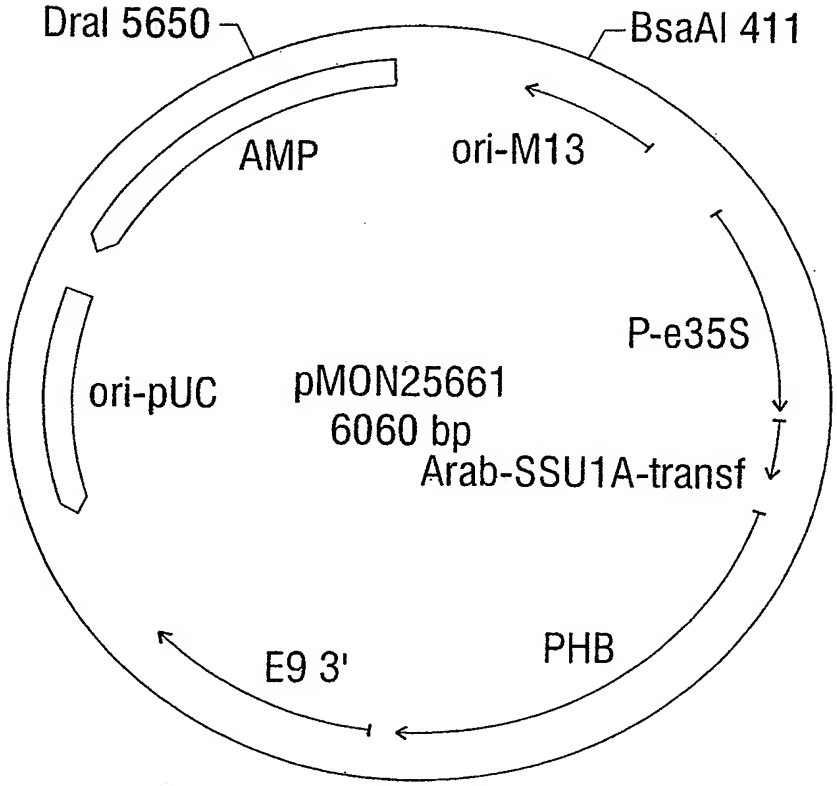
DraI 5650
BsaAI 411
AMP
ori-M13
P-e35S
ori-pUC
pMON25661
6060 bp
Arab-SSU1A-transf
E9 3'
PHB

FIG. 6

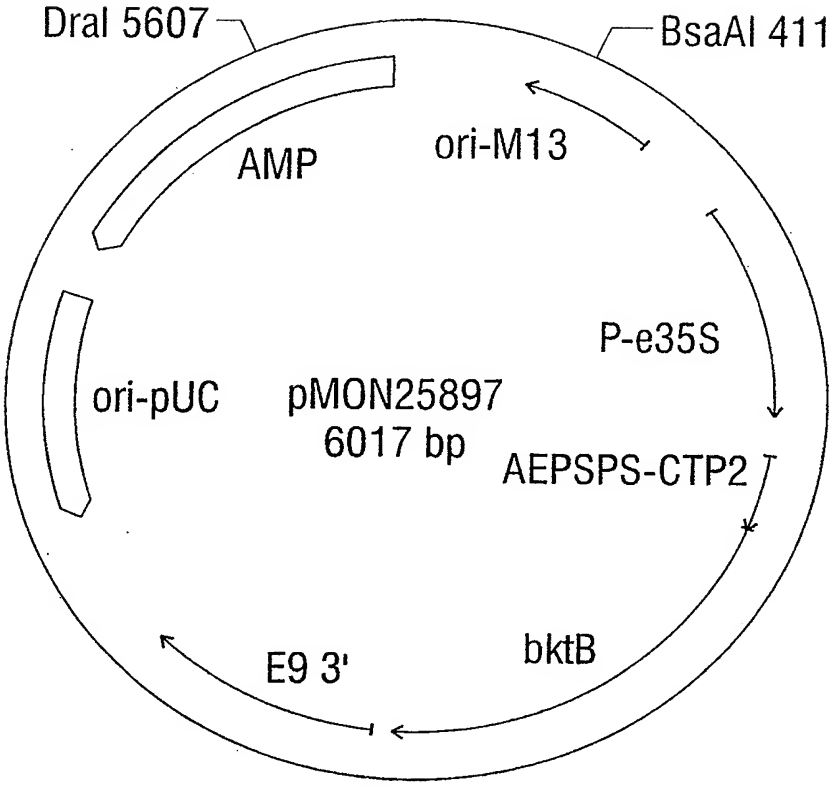
DraI 5607
BsaAI 411
AMP
ori-M13
P-e35S
ori-pUC
pMON25897
6017 bp
AEPSPS-CTP2
E9 3'
bktB

FIG. 7

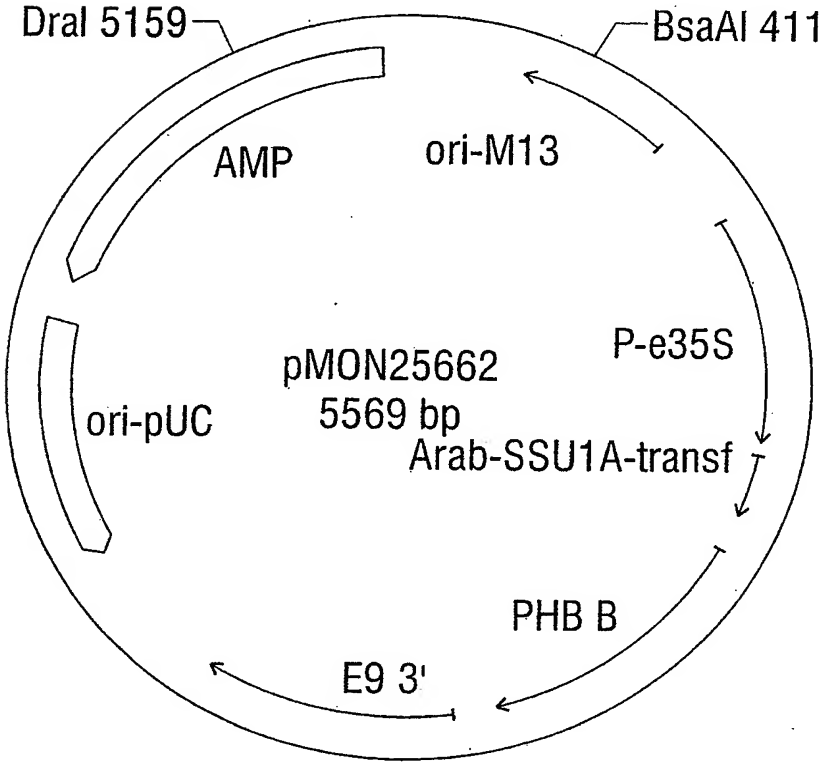
DraI 5159
BsaAI 411
AMP
ori-M13
P-e35S
pMON25662
5569 bp
ori-pUC
Arab-SSU1A-transf
PHB B
E9 3'

FIG. 8

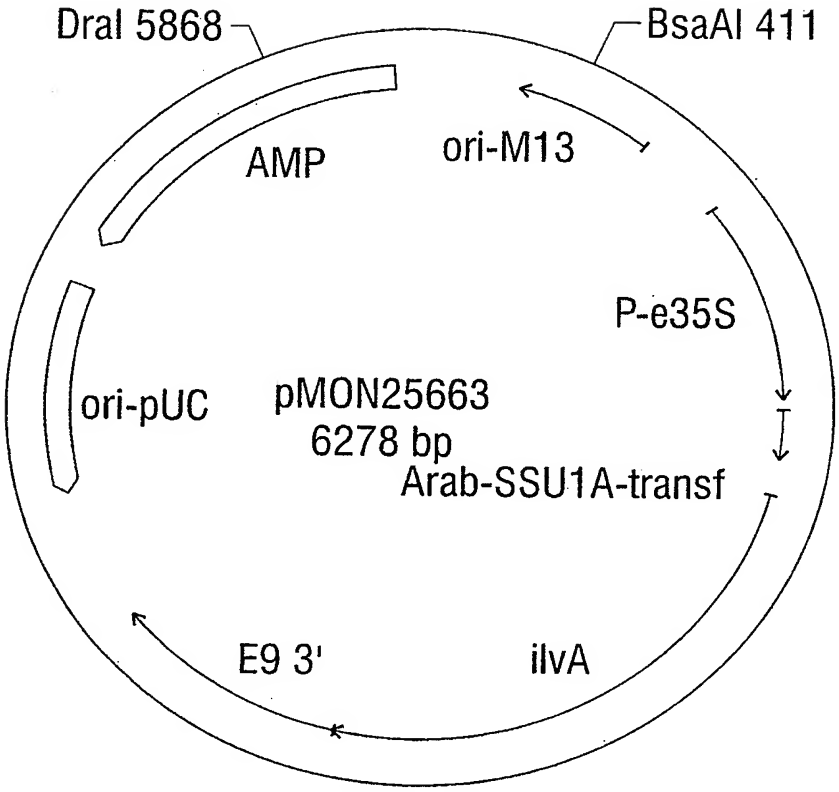
DraI 5868
BsaAI 411
AMP
ori-M13
P-e35S
ori-pUC
pMON25663
6278 bp
Arab-SSU1A-transf
E9 3'
ilvA

FIG. 9

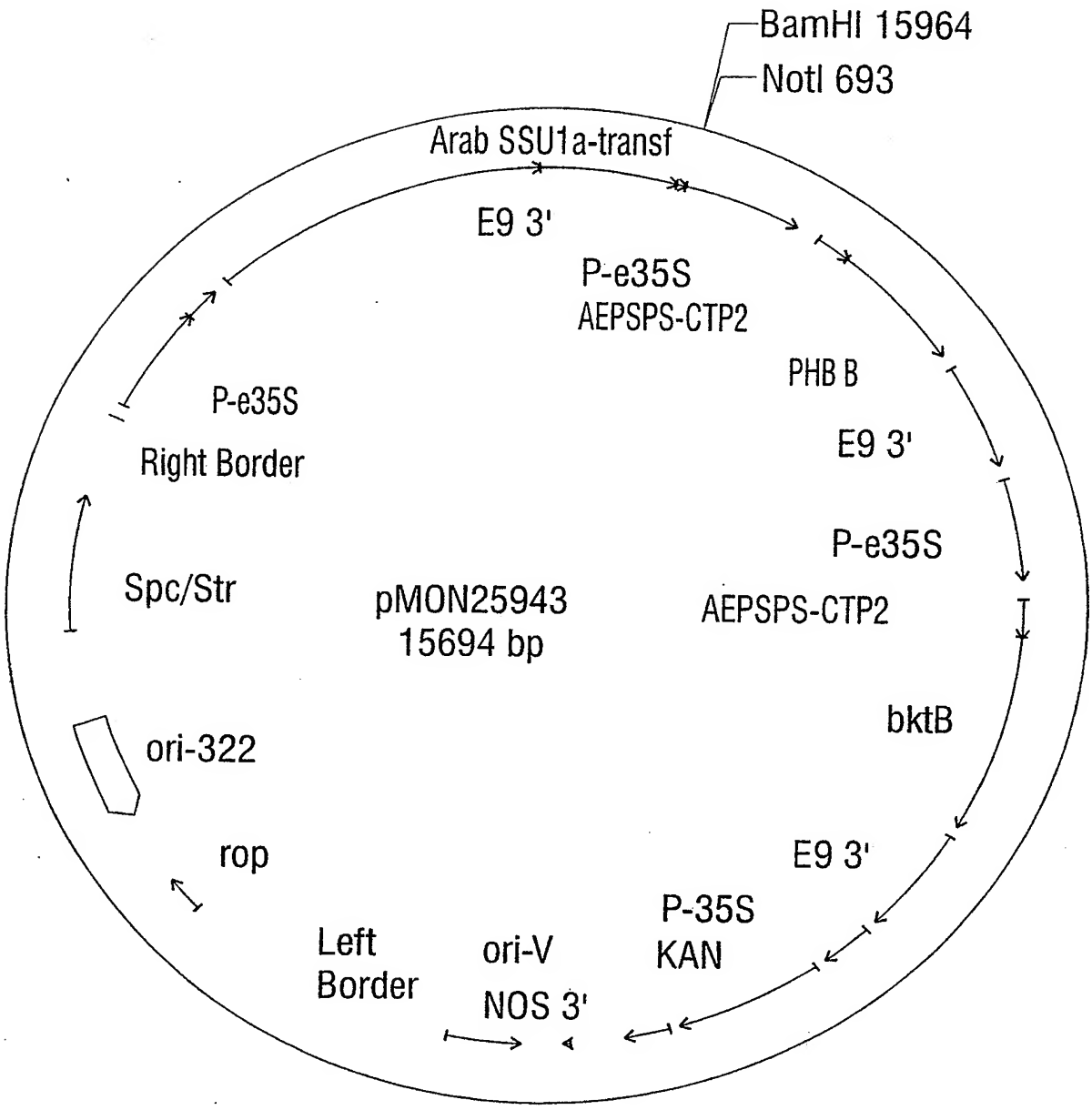
BamHI 15964
NotI 693
Arab SSU1a-transf
E9 3'
P-e35S
AEPSPS-CTP2
PHB B
E9 3'
P-e35S
AEPSPS-CTP2
bktB
E9 3'
P-35S
KAN
NOS 3'
ori-V
Left
Border
rop
ori-322
Spc/Str
P-e35S
Right Border
pMON25943
15694 bp

FIG. 10

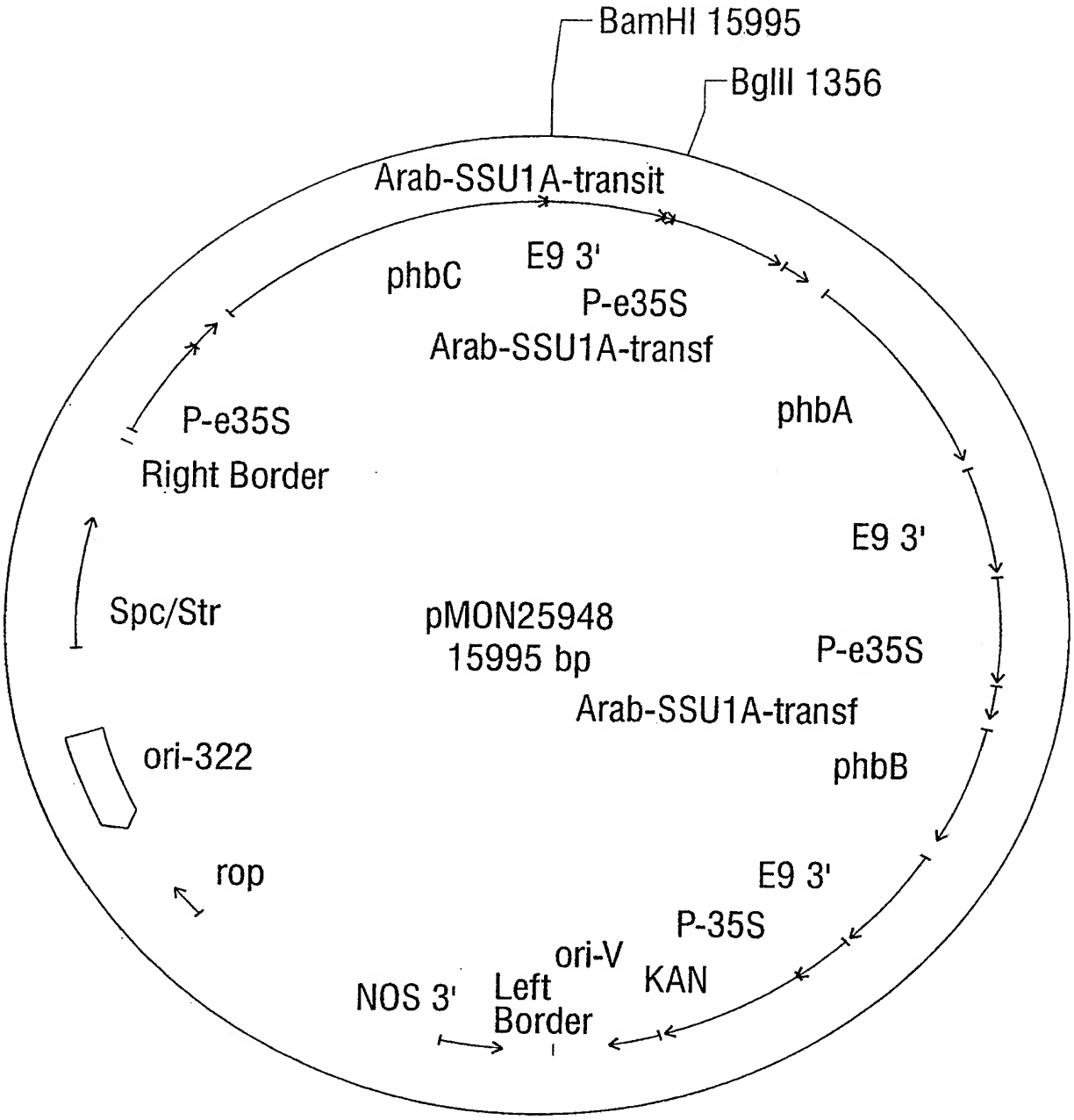
BamHI 15995
BglII 1356
Arab-SSU1A-transit
E9 3'
phbC
P-e35S
Arab-SSU1A-transf
P-e35S
Right Border
phbA
E9 3'
Spc/Str
pMON25948
15995 bp
P-e35S
Arab-SSU1A-transf
ori-322
phbB
rop
E9 3'
P-35S
ori-V
KAN
NOS 3'
Left
Border

FIG. 11

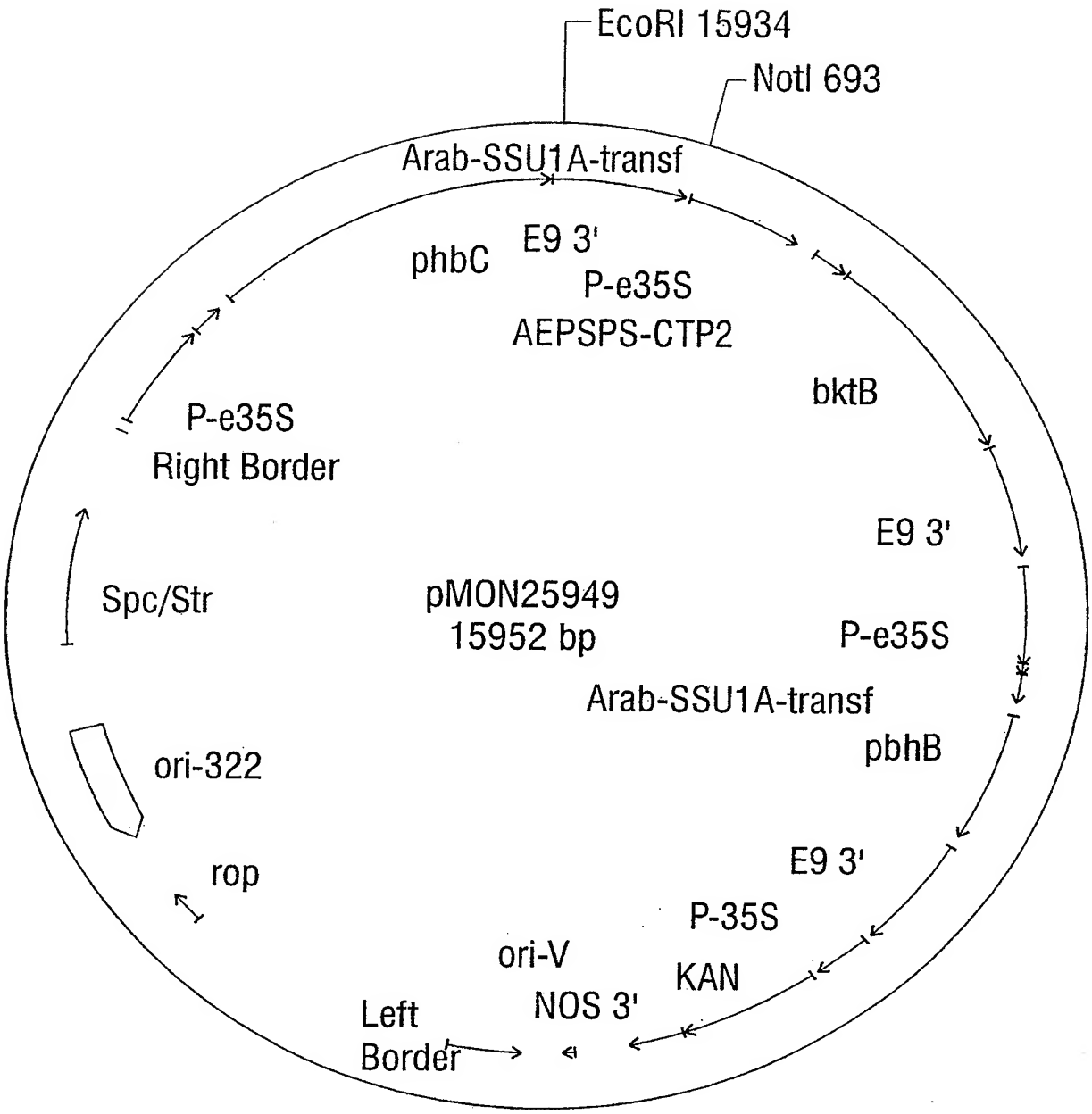
EcoRI 15934
NotI 693
Arab-SSU1A-transf
phbC
E9 3'
P-e35S
AEPSPS-CTP2
bktB
P-e35S
Right Border
E9 3'
Spc/Str
pMON25949
15952 bp
P-e35S
Arab-SSU1A-transf
pbhB
ori-322
E9 3'
rop
P-35S
ori-V
KAN
NOS 3'
Left
Border

FIG. 12

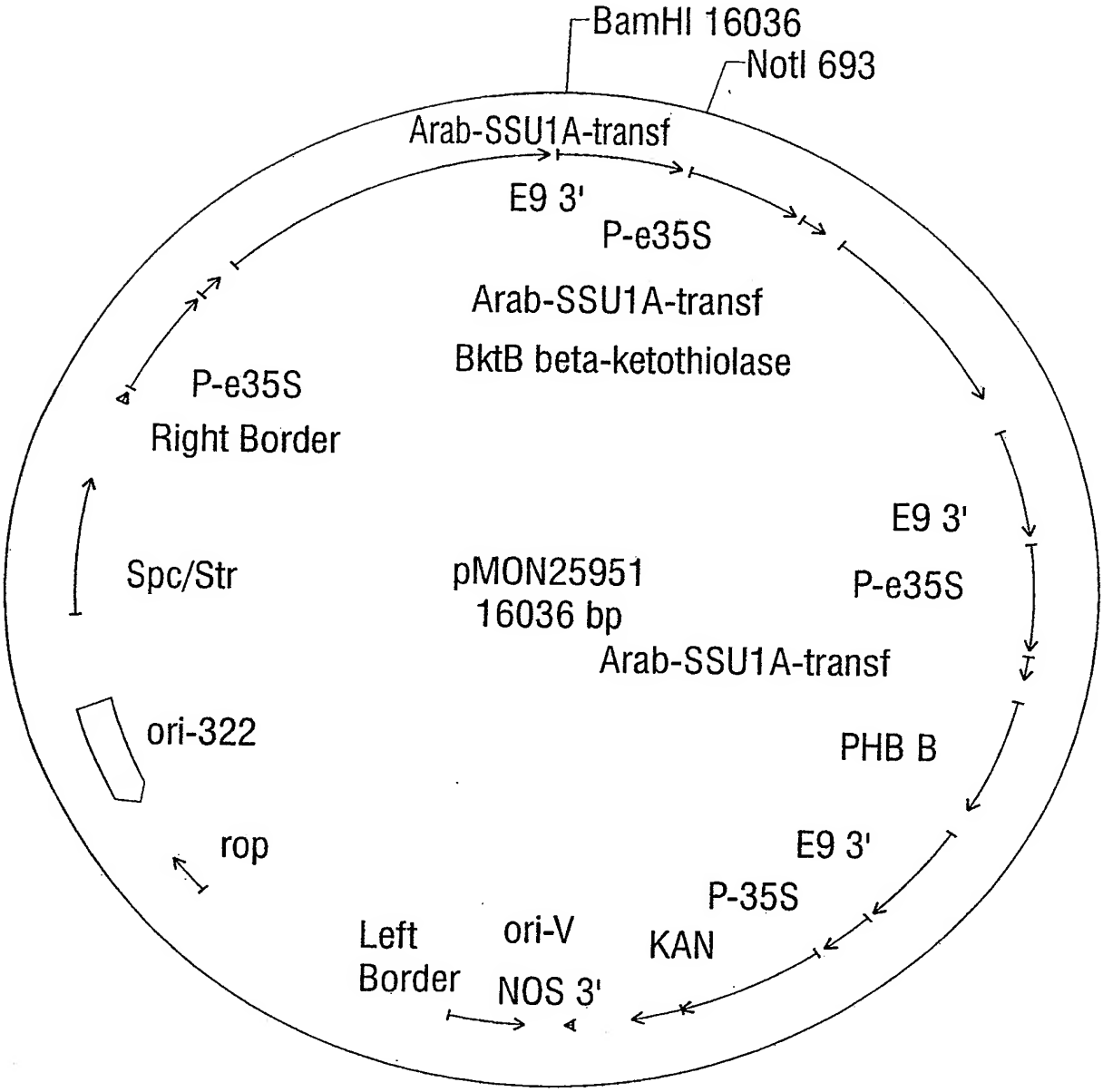
BamHI 16036
NotI 693
Arab-SSU1A-transf
E9 3'
P-e35S
Arab-SSU1A-transf
BktB beta-ketothiolase
P-e35S
Right Border
E9 3'
Spc/Str
pMON25951
16036 bp
P-e35S
Arab-SSU1A-transf
ori-322
PHB B
rop
E9 3'
P-35S
Left
Border
ori-V
KAN
NOS 3'

FIG. 13

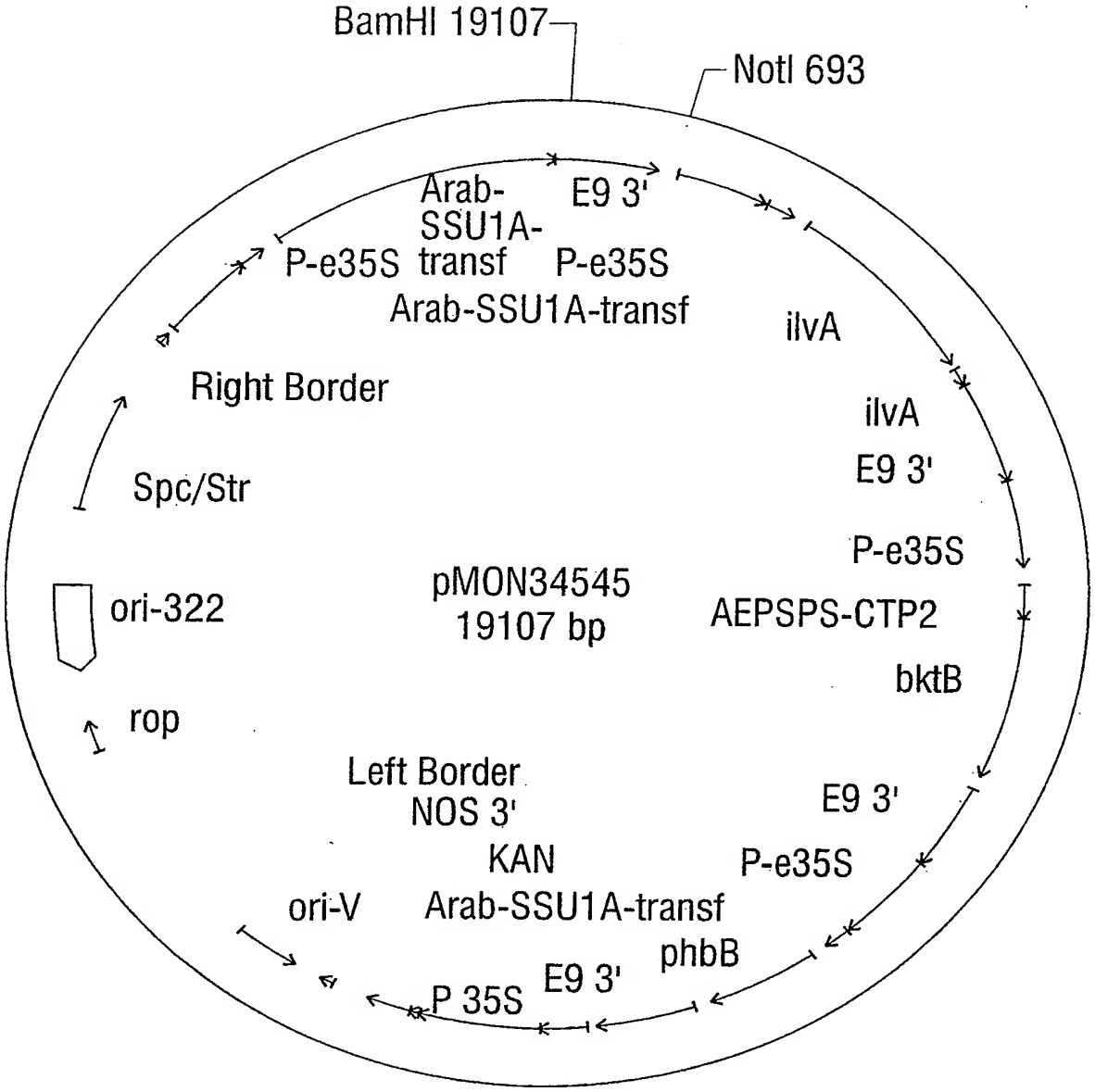
BamHI 19107
NotI 693
Arab-SSU1A-transf
E9 3'
P-e35S
Arab-SSU1A-transf
P-e35S
ilvA
Right Border
ilvA
E9 3'
Spc/Str
P-e35S
pMON34545
19107 bp
ori-322
AEPSPS-CTP2
bktB
rop
Left Border
NOS 3'
E9 3'
KAN
P-e35S
ori-V
Arab-SSU1A-transf
phbB
E9 3'
P 35S

FIG. 14

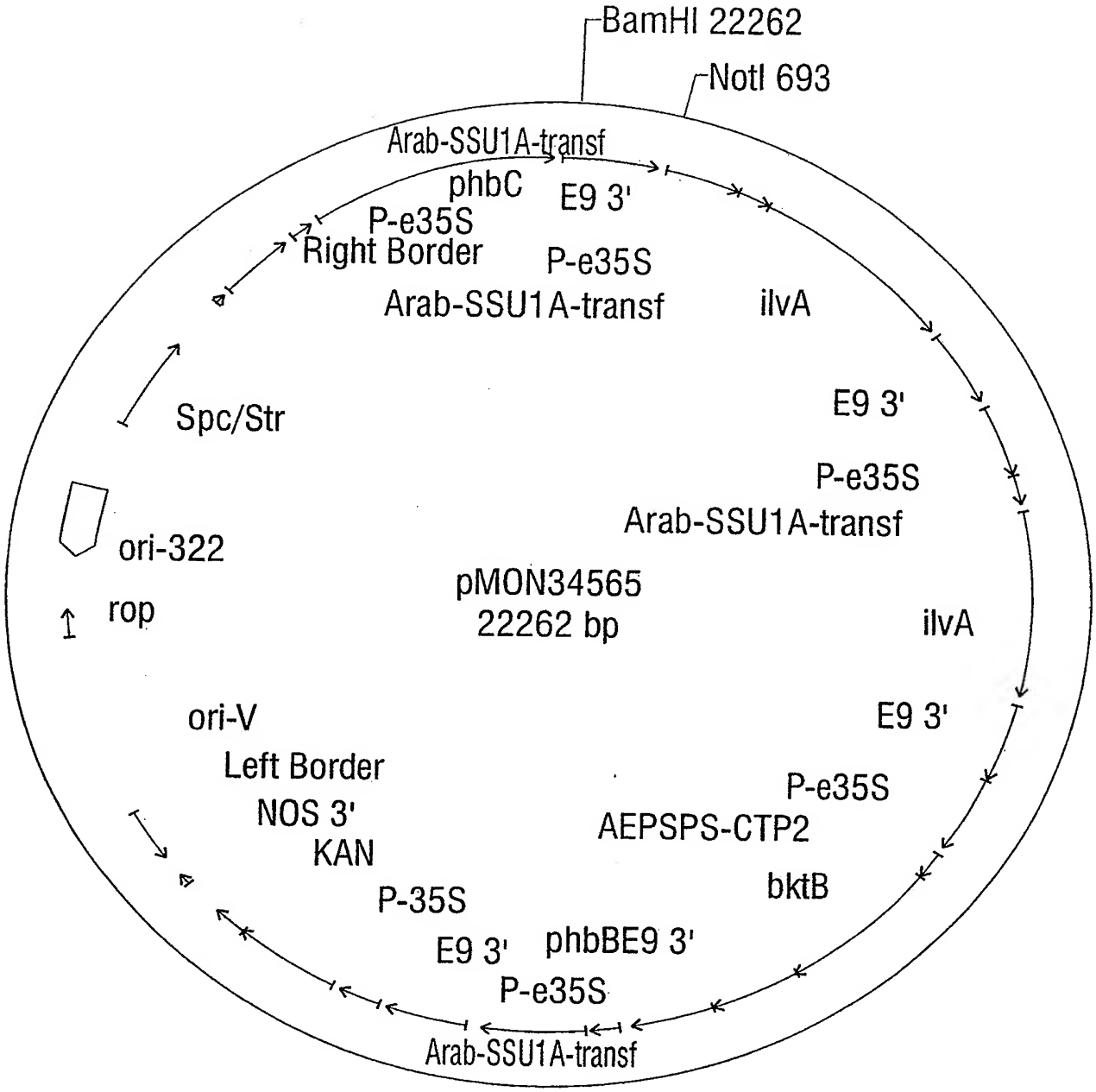
BamHI 22262
NotI 693
Arab-SSU1A-transf
phbC
E9 3'
P-e35S
Right Border
P-e35S
Arab-SSU1A-transf
ilvA
E9 3'
P-e35S
Arab-SSU1A-transf
ilvA
E9 3'
P-e35S
AEPSPS-CTP2
bktB
phbBE9 3'
P-e35S
Arab-SSU1A-transf
E9 3'
P-35S
KAN
NOS 3'
Left Border
ori-V
rop
ori-322
Spc/Str
pMON34565
22262 bp

FIG. 15

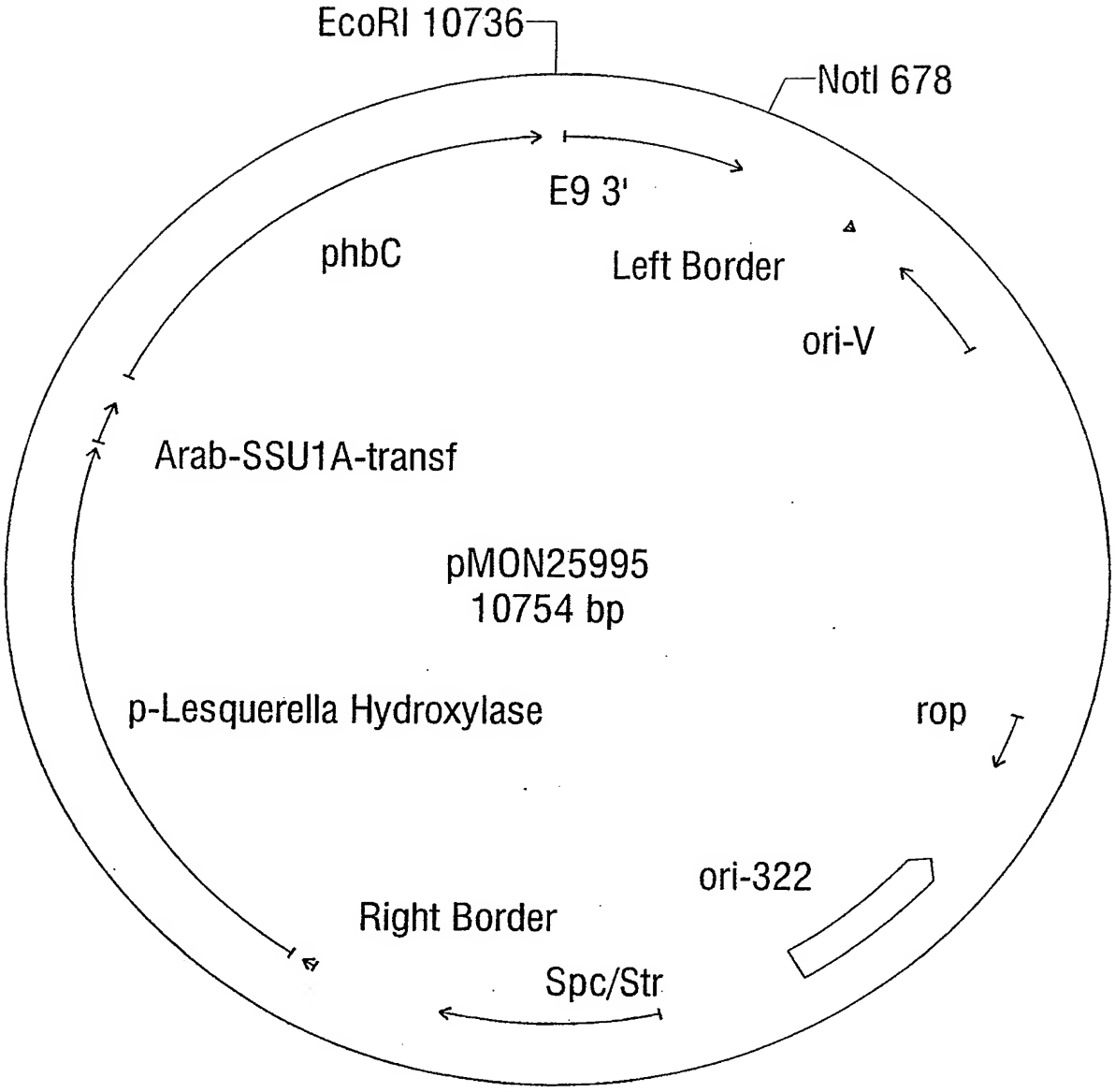
EcoRI 10736
NotI 678
E9 3'
phbC
Left Border
ori-V
Arab-SSU1A-transf
pMON25995
10754 bp
p-Lesquerella Hydroxylase
rop
ori-322
Right Border
Spc/Str

FIG. 16

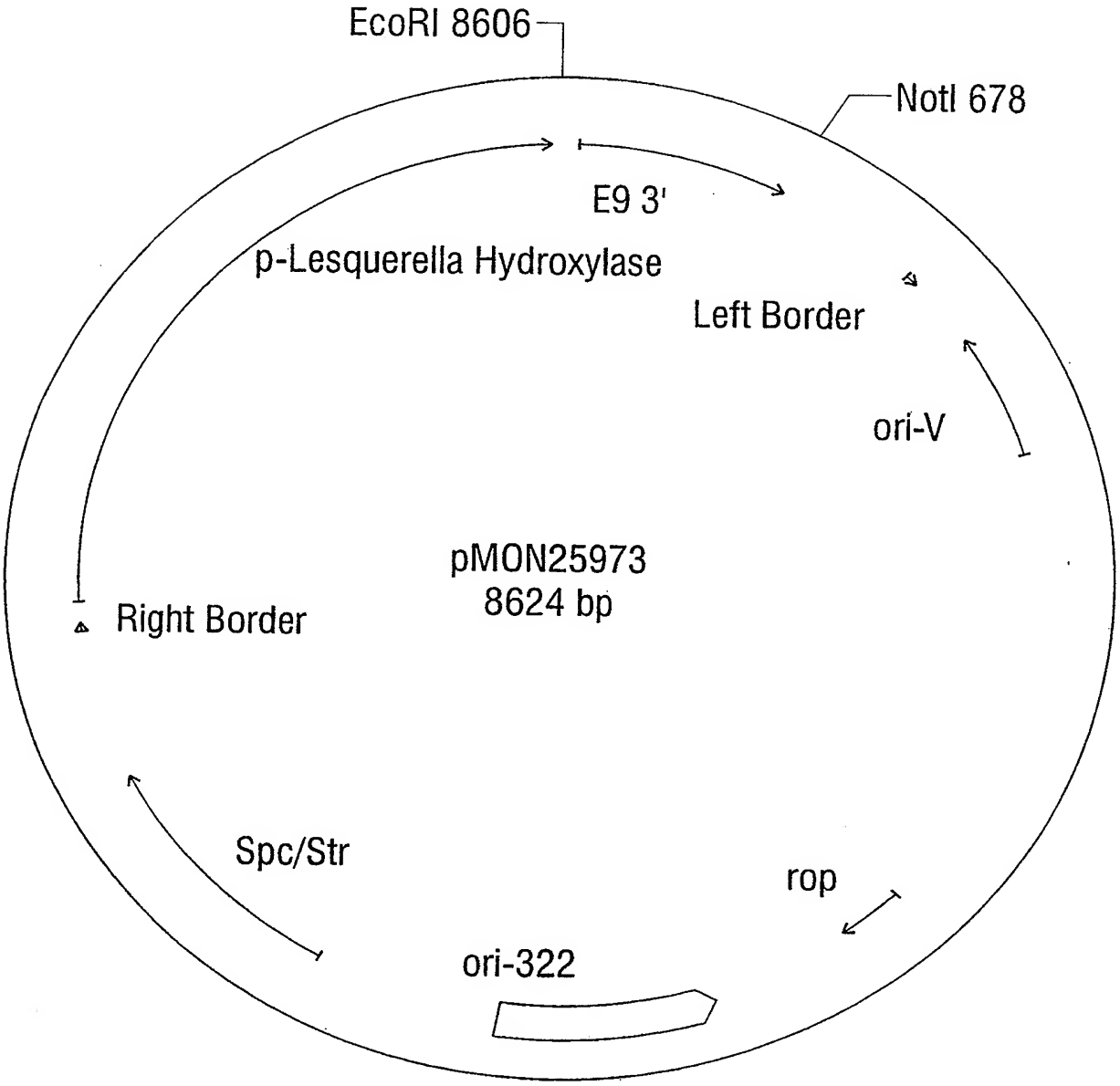
EcoRI 8606
NotI 678
E9 3'
p-Lesquerella Hydroxylase
Left Border
ori-V
pMON25973
8624 bp
Right Border
Spc/Str
rop
ori-322

FIG. 17

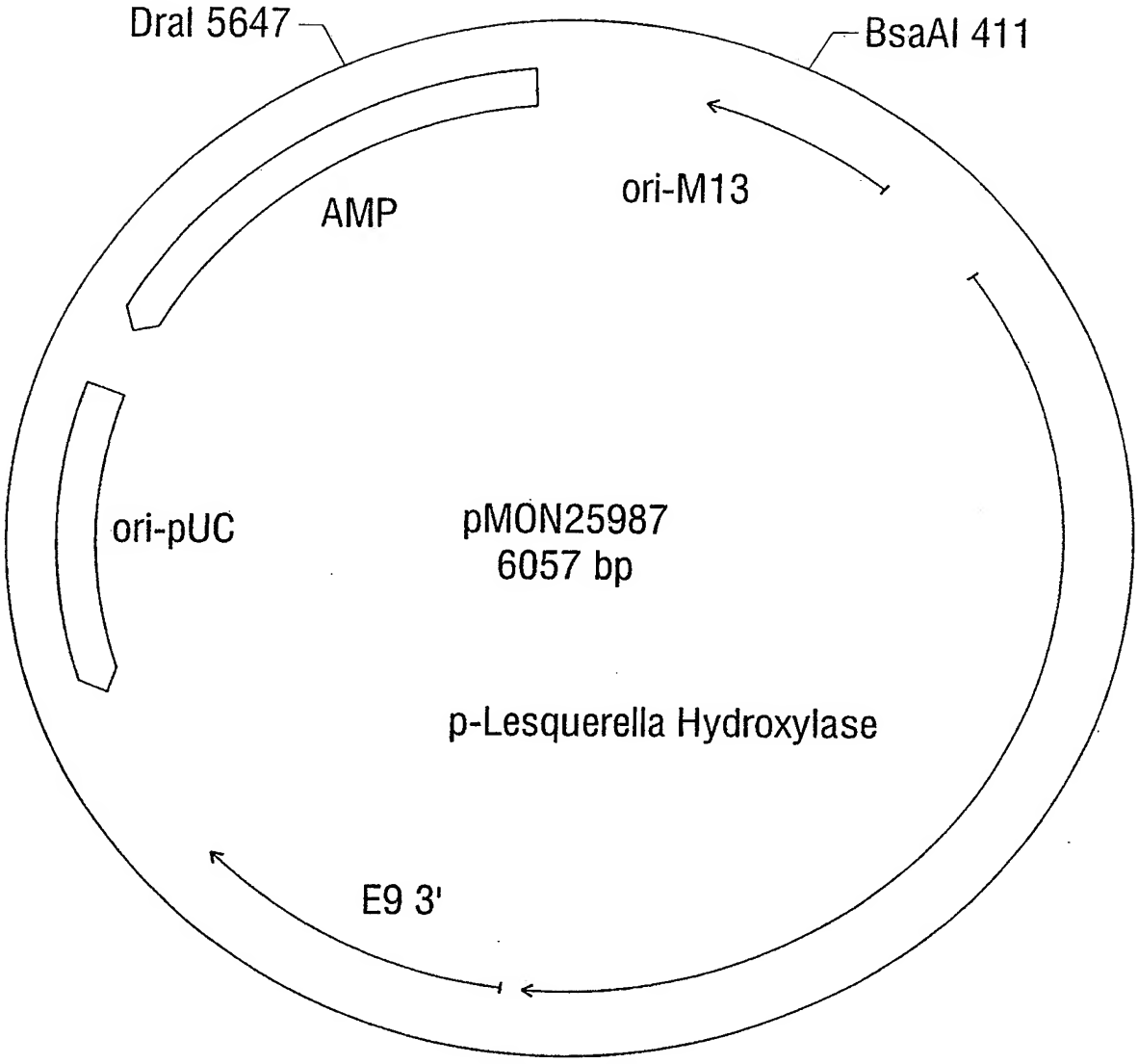
DraI 5647
BsaAI 411
AMP
ori-M13
ori-pUC
pMON25987
6057 bp
p-Lesquerella Hydroxylase
E9 3'

FIG. 18

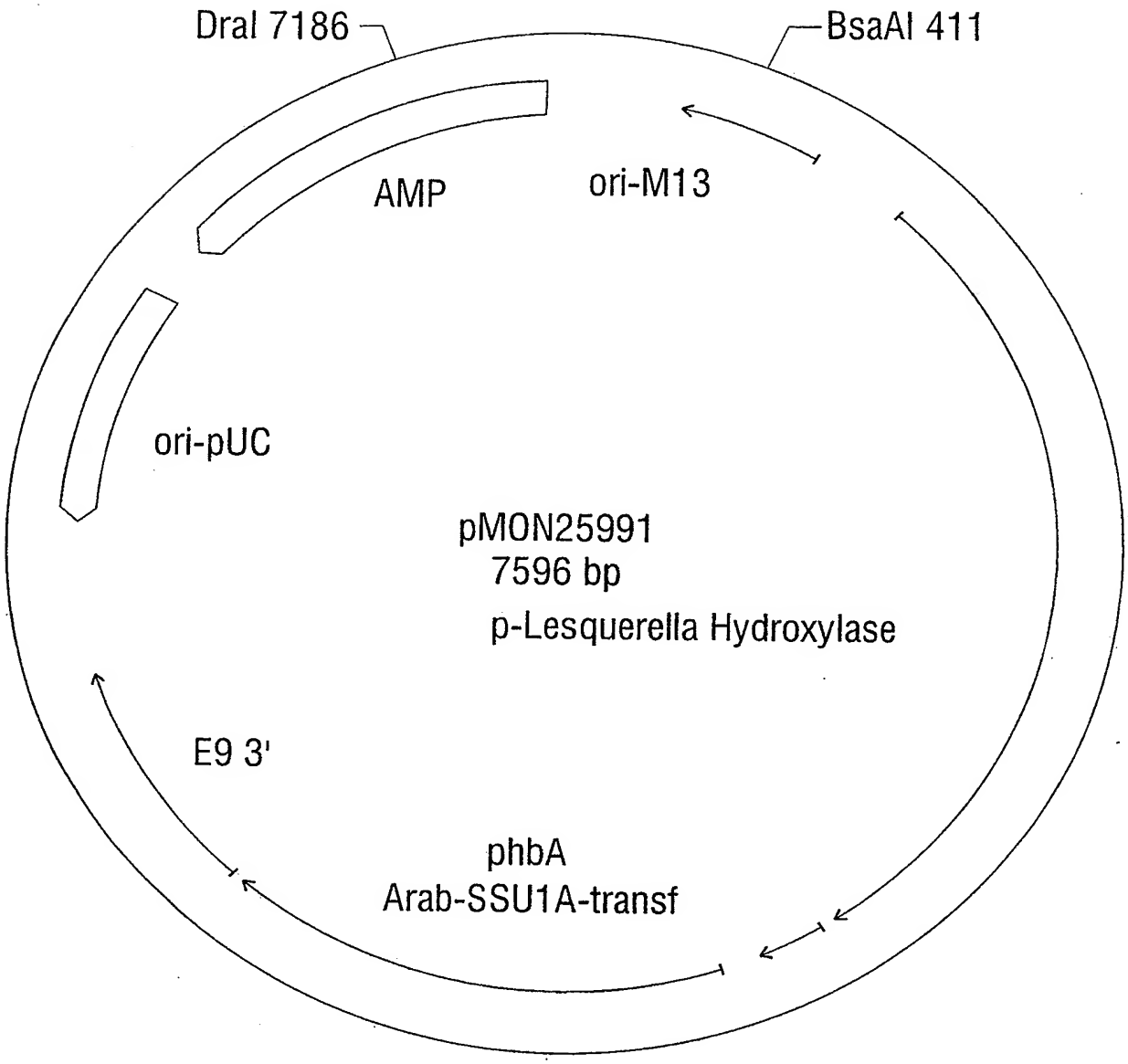
DraI 7186
BsaAI 411
AMP
ori-M13
ori-pUC
pMON25991
7596 bp
p-Lesquerella Hydroxylase
E9 3'
phbA
Arab-SSU1A-transf

FIG. 19

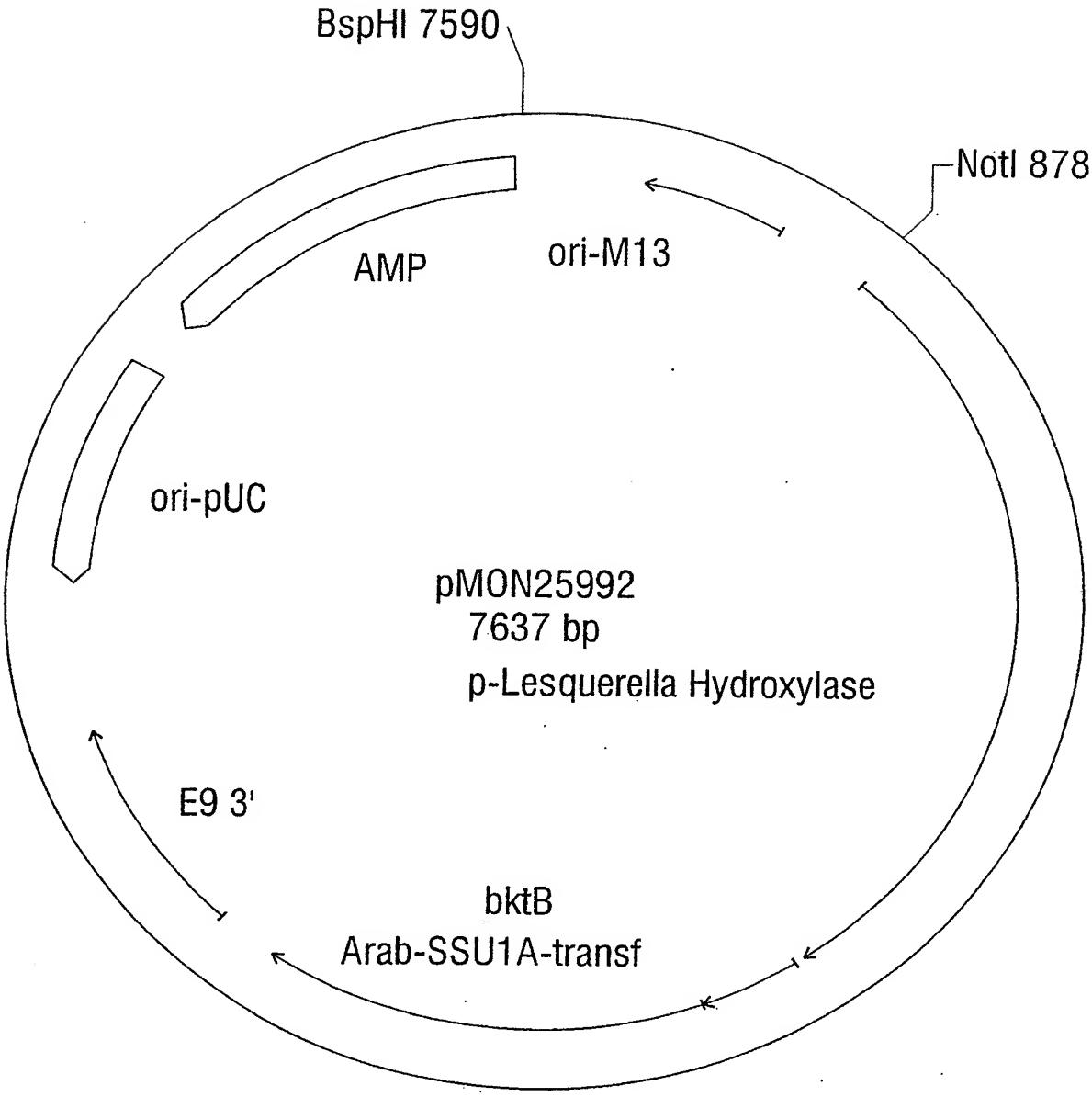
BspHI 7590
NotI 878
AMP
ori-M13
ori-pUC
pMON25992
7637 bp
p-Lesquerella Hydroxylase
E9 3'
bktB
Arab-SSU1A-transf

FIG. 20

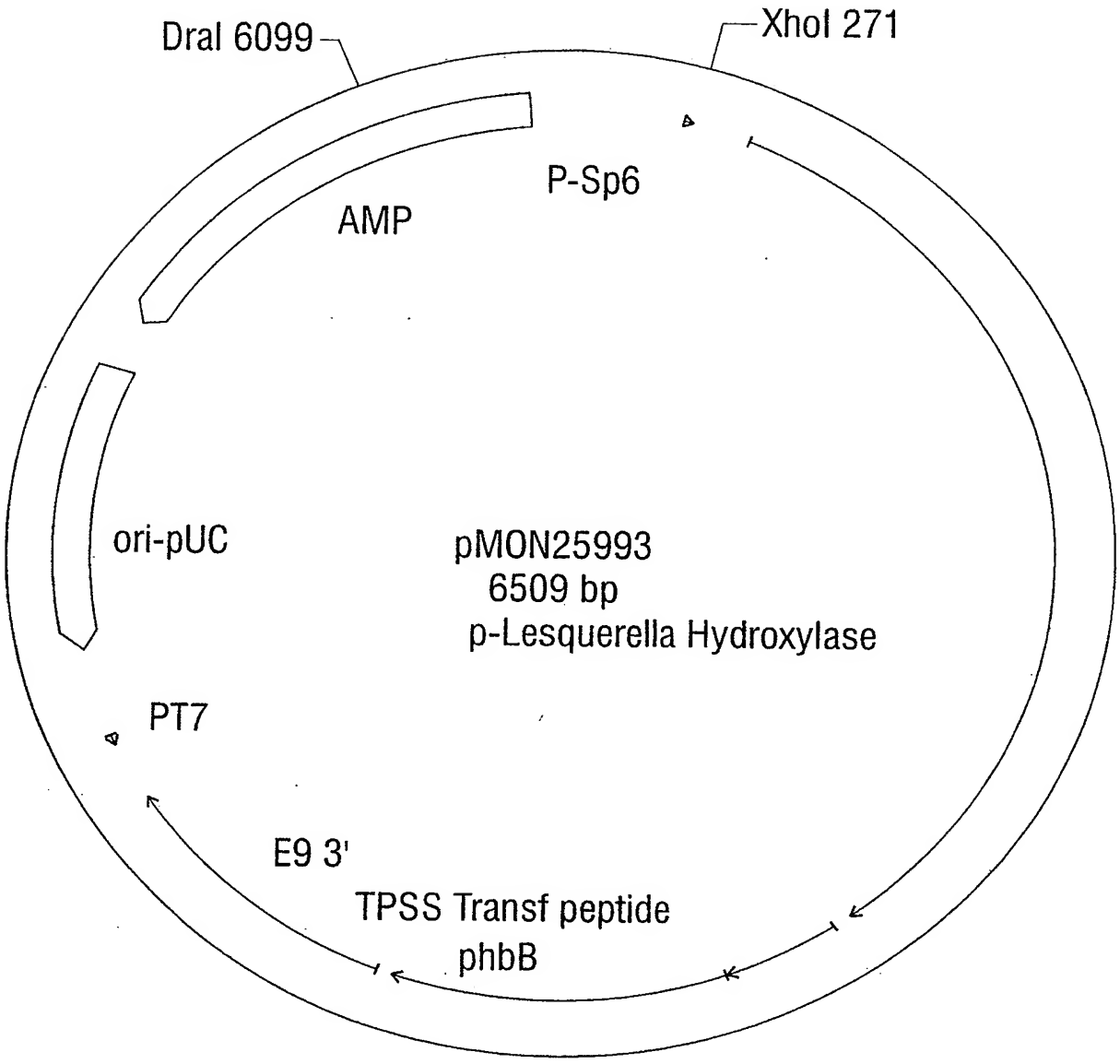
DraI 6099
XhoI 271
P-Sp6
AMP
ori-pUC
pMON25993
6509 bp
p-Lesquerella Hydroxylase
PT7
E9 3'
TPSS Transf peptide
phbB

FIG. 21

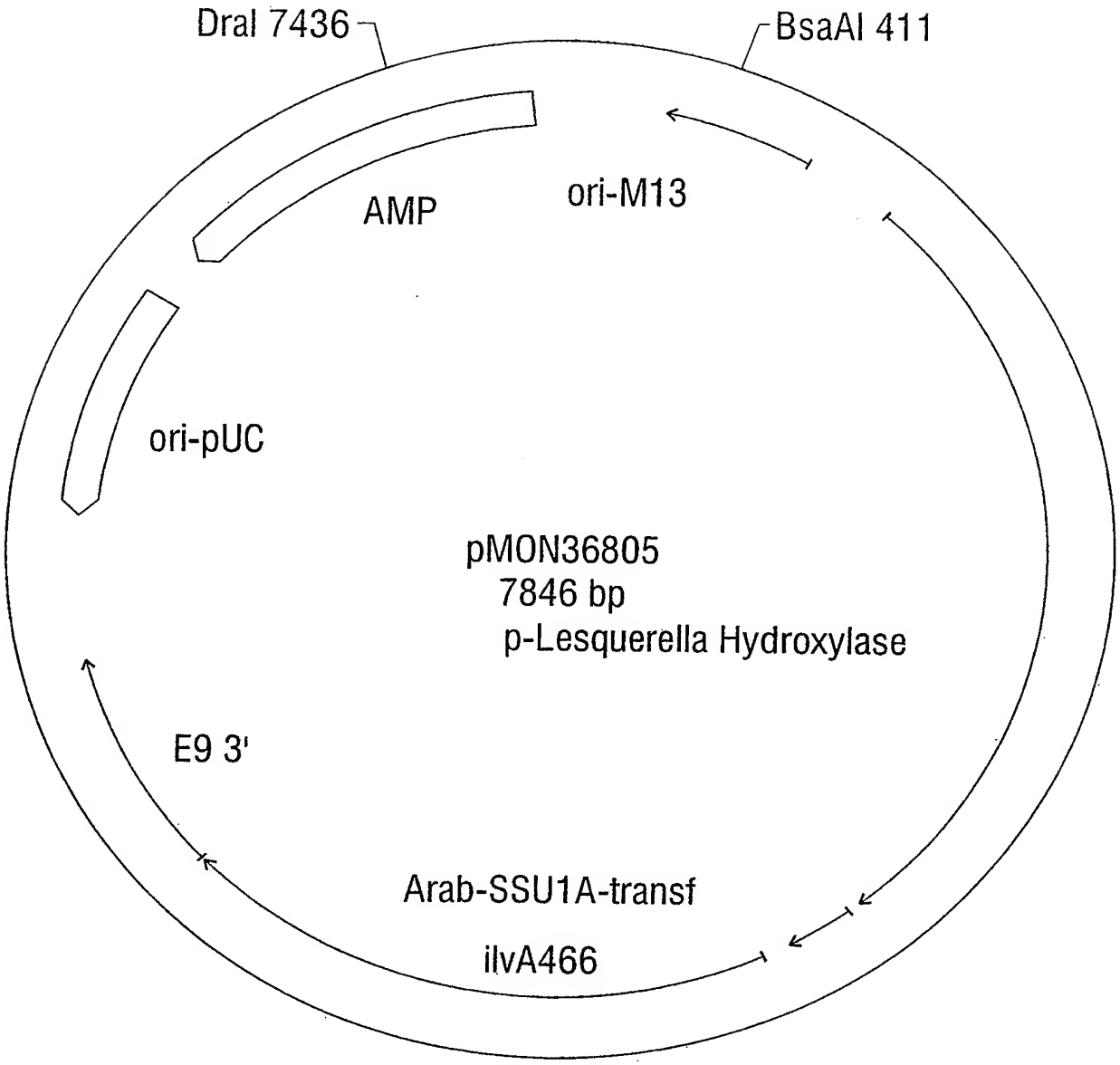
DraI 7436
BsaAI 411
AMP
ori-M13
ori-pUC
pMON36805
7846 bp
p-Lesquerella Hydroxylase
E9 3'
Arab-SSU1A-transf
ilvA466

FIG. 22

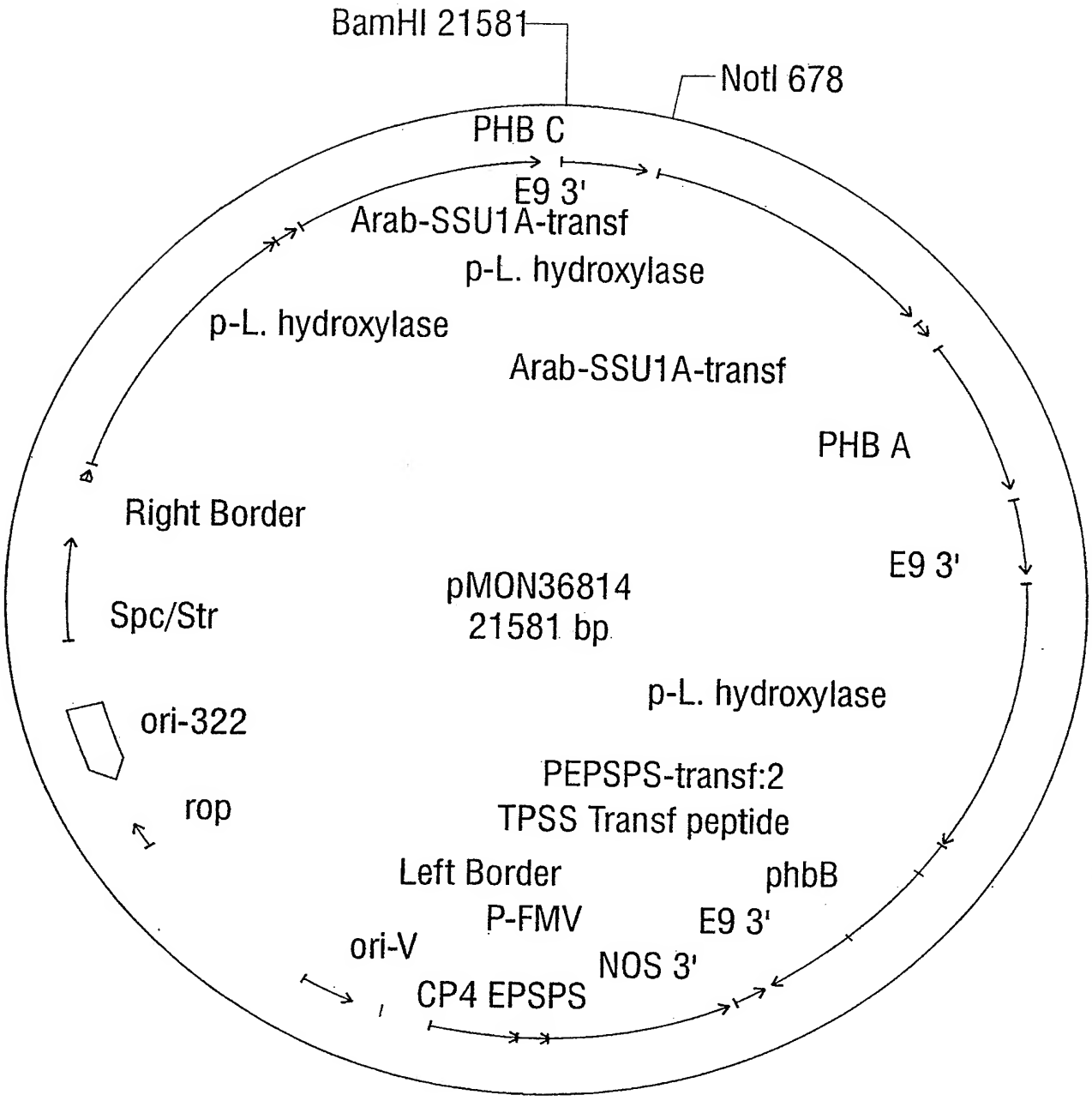
BamHI 21581
NotI 678
PHB C
E9 3'
Arab-SSU1A-transf
p-L. hydroxylase
p-L. hydroxylase
Arab-SSU1A-transf
PHB A
Right Border
E9 3'
pMON36814
21581 bp
Spc/Str
p-L. hydroxylase
ori-322
PEPSPS-transf:2
rop
TPSS Transf peptide
Left Border
phbB
P-FMV
E9 3'
ori-V
NOS 3'
CP4 EPSPS

FIG. 23

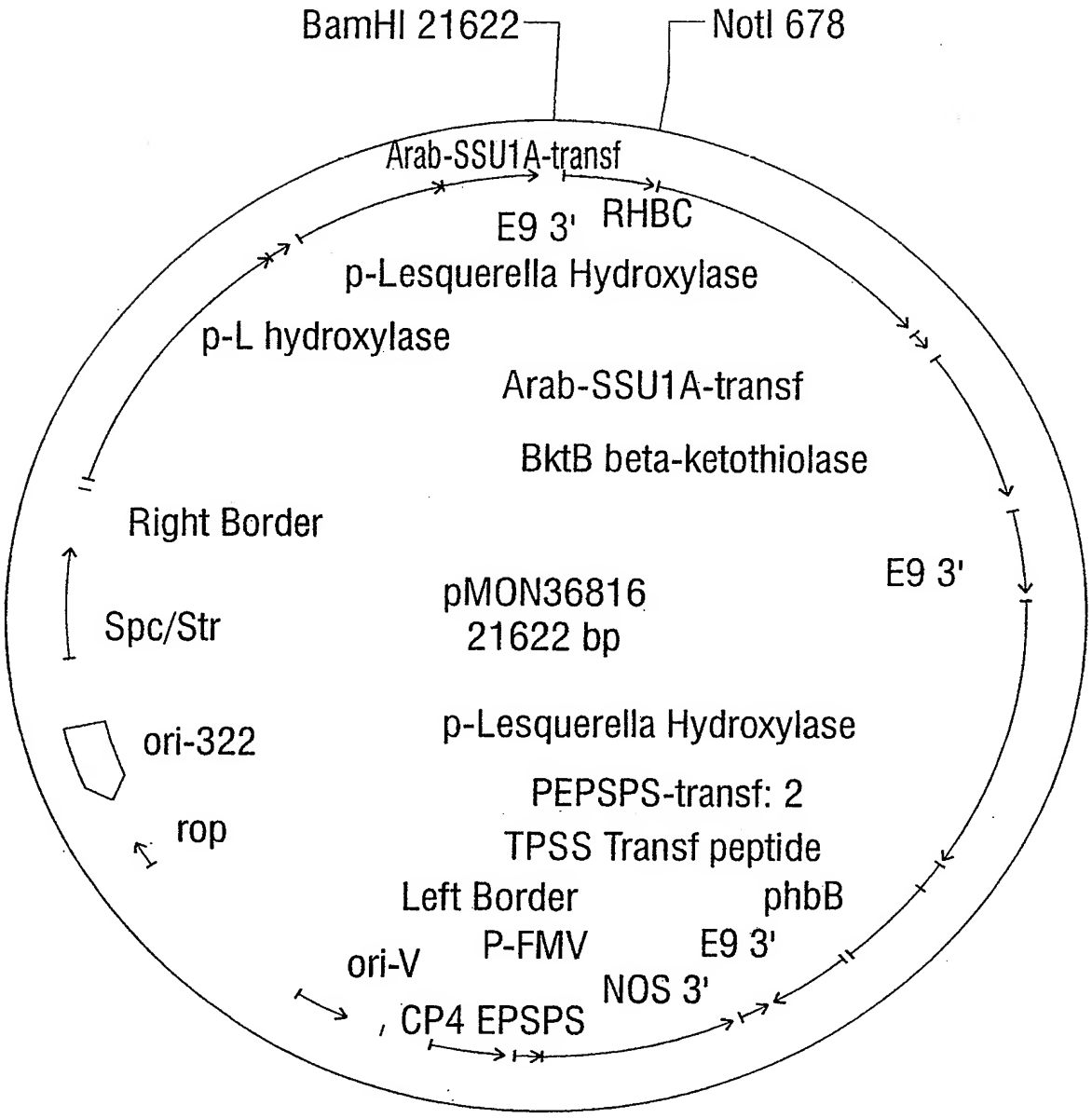
BamHI 21622
NotI 678
Arab-SSU1A-transf
E9 3'
RHBC
p-Lesquerella Hydroxylase
p-L hydroxylase
Arab-SSU1A-transf
BktB beta-ketothiolase
Right Border
E9 3'
pMON36816
21622 bp
Spc/Str
ori-322
p-Lesquerella Hydroxylase
PEPSPS-transf: 2
rop
TPSS Transf peptide
Left Border
phbB
P-FMV
E9 3'
ori-V
NOS 3'
CP4 EPSPS

FIG. 24

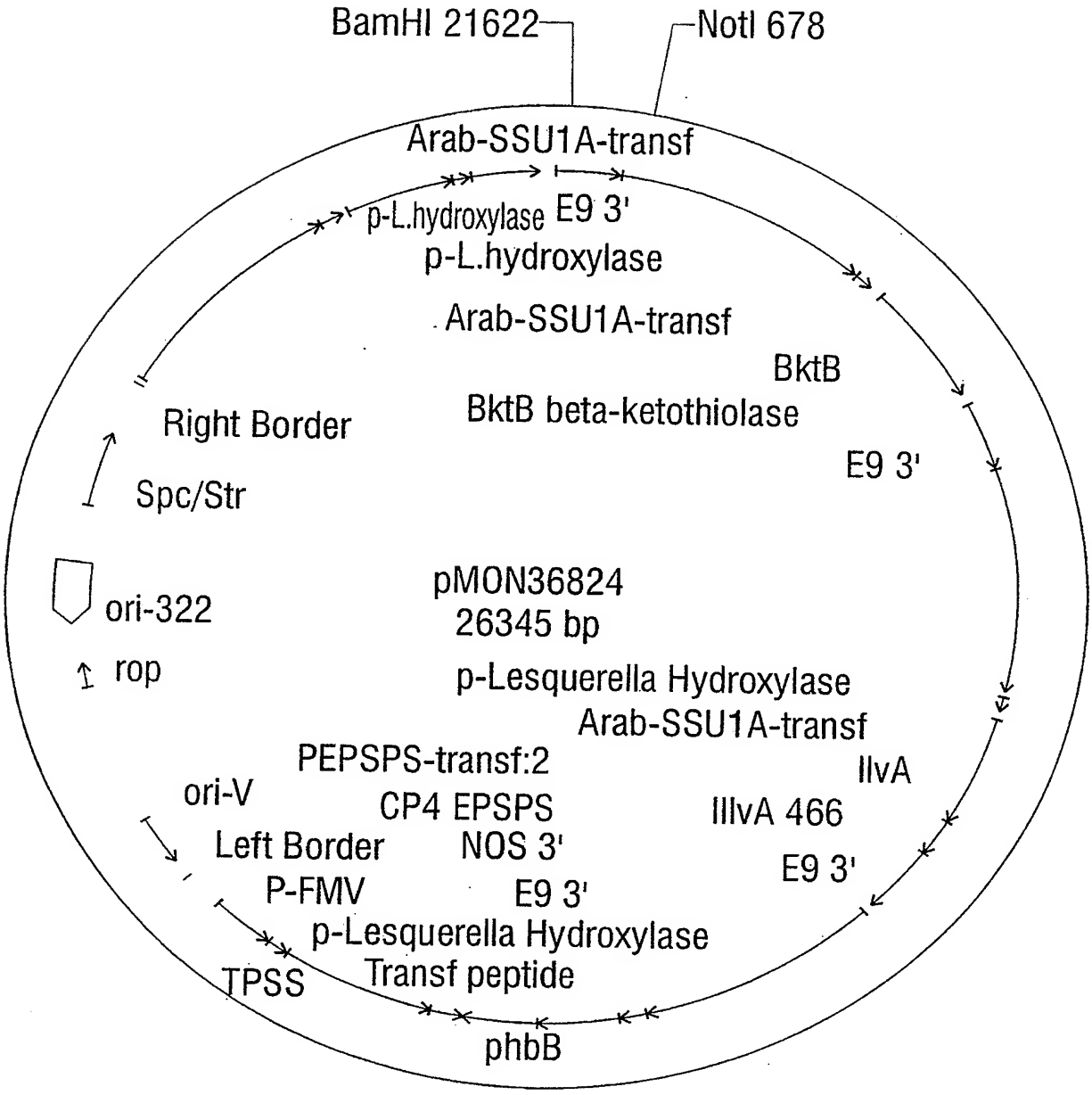
BamHI 21622
NotI 678
Arab-SSU1A-transf
p-L.hydroxylase
E9 3'
p-L.hydroxylase
Arab-SSU1A-transf
BktB
BktB beta-ketothiolase
Right Border
E9 3'
Spc/Str
pMON36824
26345 bp
ori-322
rop
p-Lesquerella Hydroxylase
Arab-SSU1A-transf
PEPSPS-transf:2
IlvA
ori-V
CP4 EPSPS
IlIvA 466
Left Border
NOS 3'
E9 3'
P-FMV
E9 3'
p-Lesquerella Hydroxylase
TPSS
Transf peptide
phbB

FIG. 25

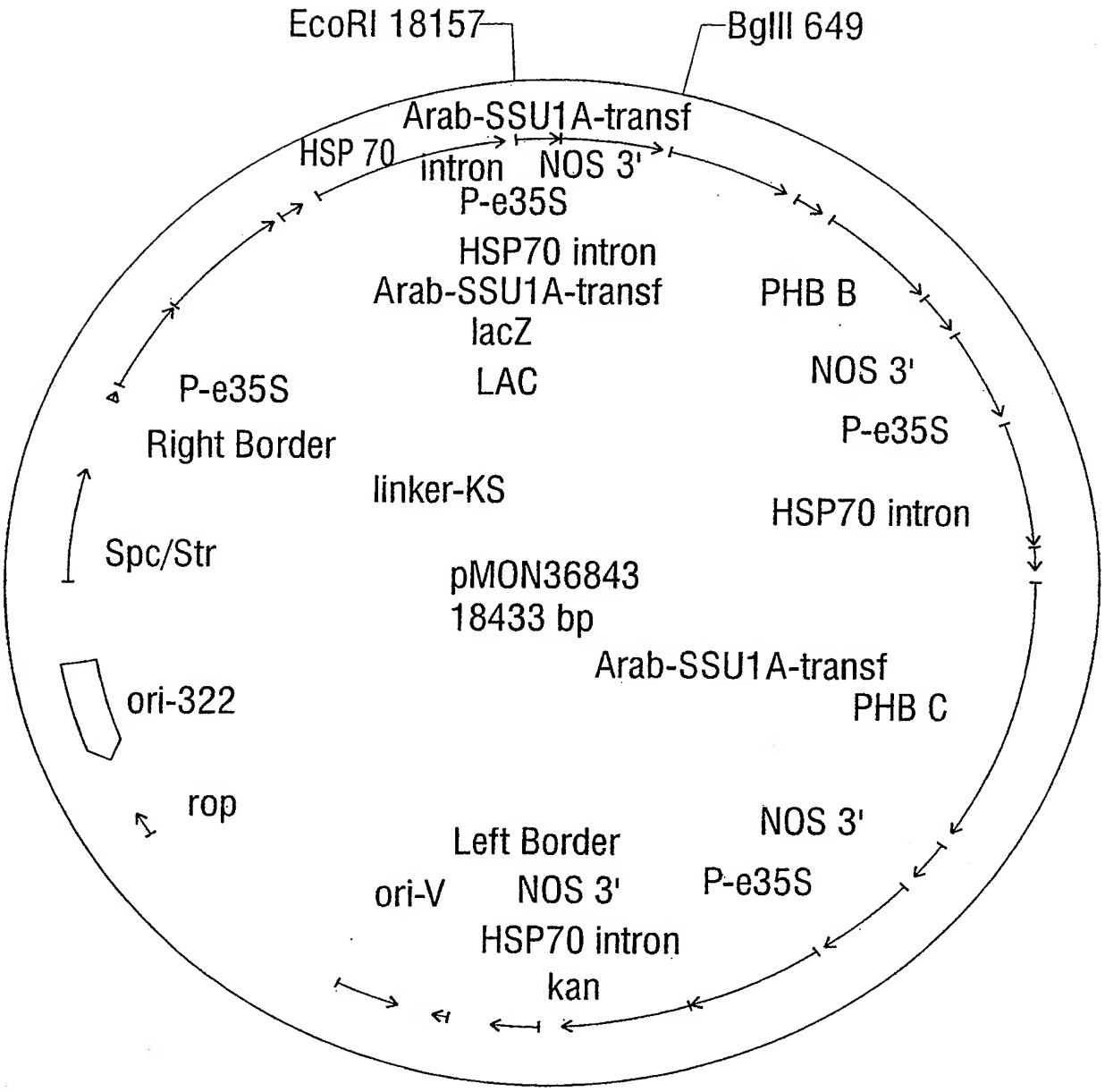
EcoRI 18157
BglII 649
Arab-SSU1A-transf
HSP 70
intron
NOS 3'
P-e35S
HSP70 intron
Arab-SSU1A-transf
lacZ
LAC
PHB B
NOS 3'
P-e35S
HSP70 intron
P-e35S
Right Border
linker-KS
Spc/Str
pMON36843
18433 bp
Arab-SSU1A-transf
PHB C
ori-322
rop
NOS 3'
Left Border
P-e35S
ori-V
NOS 3'
HSP70 intron
kan

FIG. 26

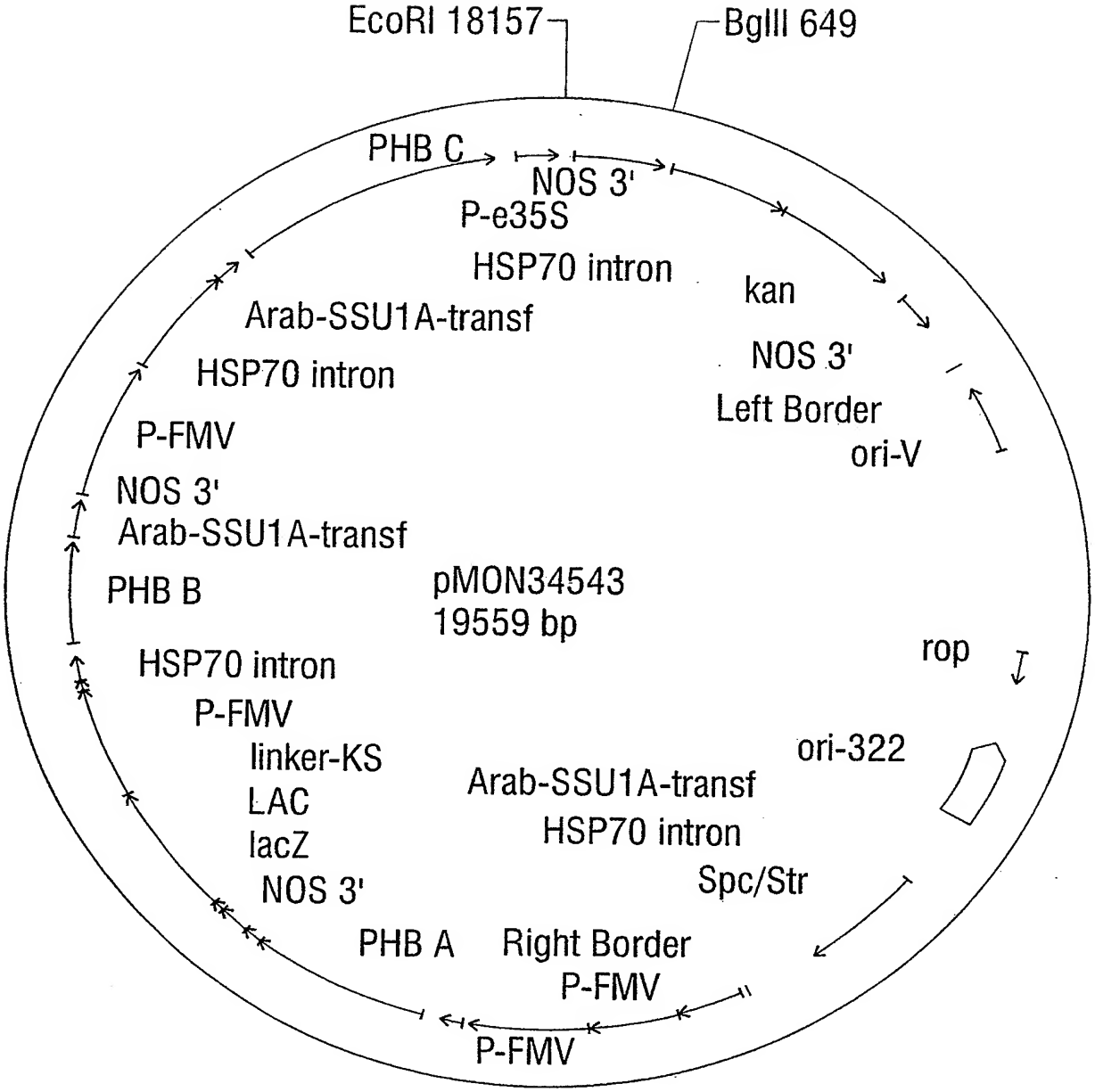
EcoRI 18157
BglII 649
PHB C
NOS 3'
P-e35S
HSP70 intron
Arab-SSU1A-transf
HSP70 intron
P-FMV
NOS 3'
Arab-SSU1A-transf
PHB B
HSP70 intron
P-FMV
linker-KS
LAC
lacZ
NOS 3'
PHB A
Right Border
P-FMV
P-FMV
pMON34543
19559 bp
kan
NOS 3'
Left Border
ori-V
rop
ori-322
Arab-SSU1A-transf
HSP70 intron
Spc/Str

FIG. 27

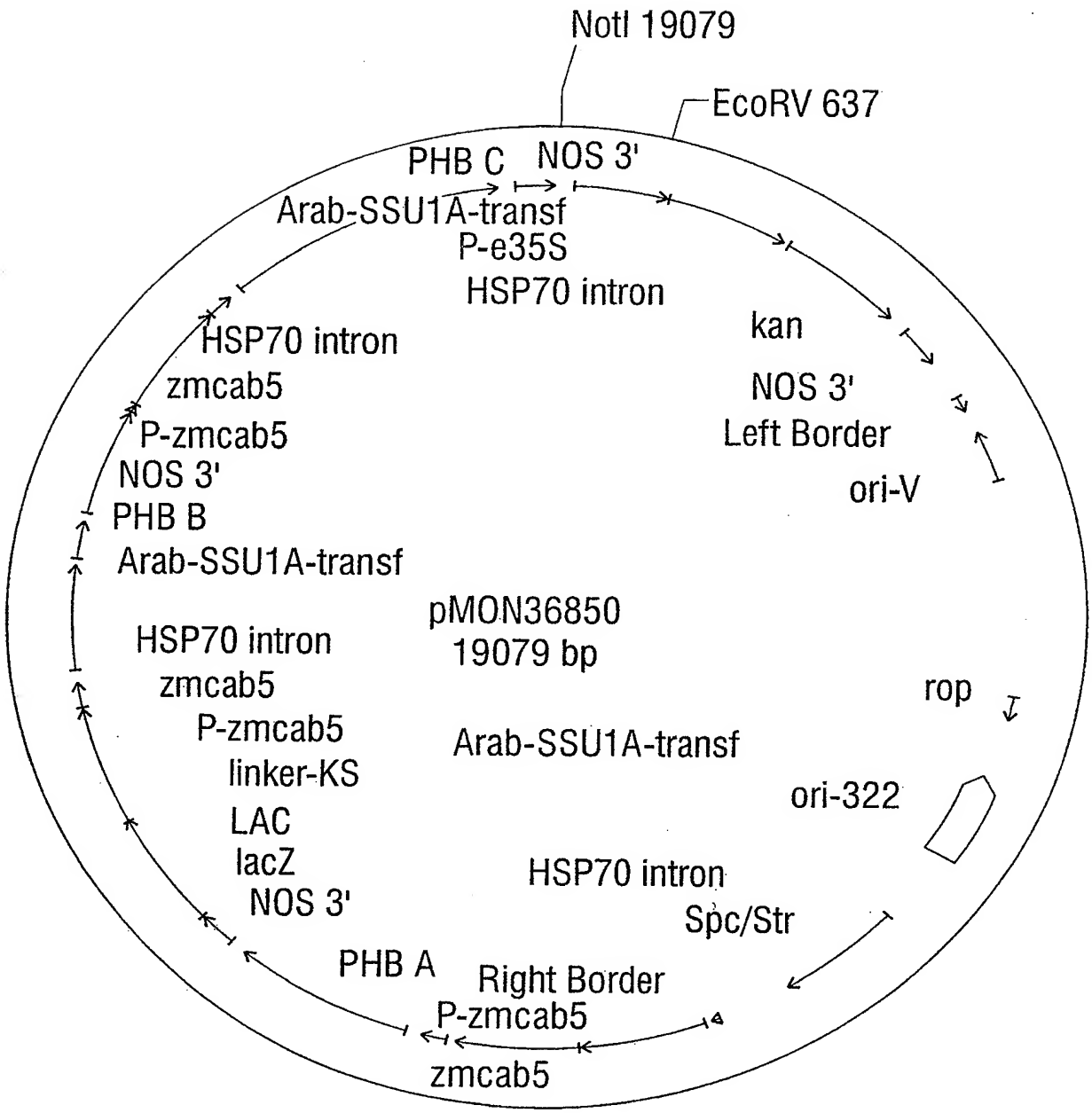
NotI 19079
EcoRV 637
PHB C
NOS 3'
Arab-SSU1A-transf
P-e35S
HSP70 intron
kan
NOS 3'
Left Border
ori-V
rop
ori-322
Spc/Str
Right Border
P-zmcab5
zmcab5
PHB A
NOS 3'
lacZ
LAC
linker-KS
P-zmcab5
zmcab5
HSP70 intron
Arab-SSU1A-transf
PHB B
NOS 3'
P-zmcab5
zmcab5
HSP70 intron
pMON36850
19079 bp
Arab-SSU1A-transf
HSP70 intron

FIG. 28

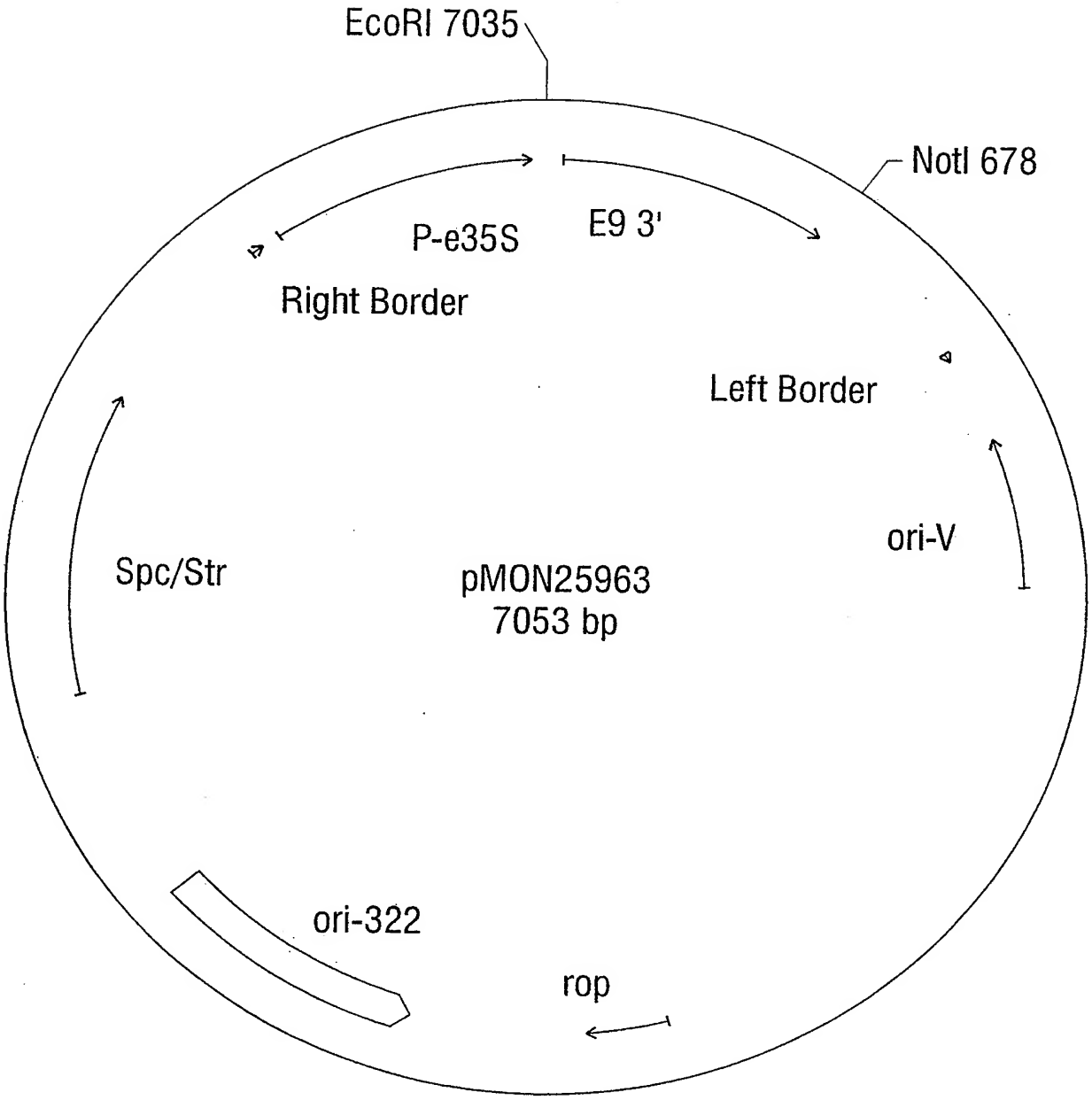
EcoRI 7035
NotI 678
P-e35S
E9 3'
Right Border
Left Border
ori-V
Spc/Str
pMON25963
7053 bp
ori-322
rop

FIG. 29

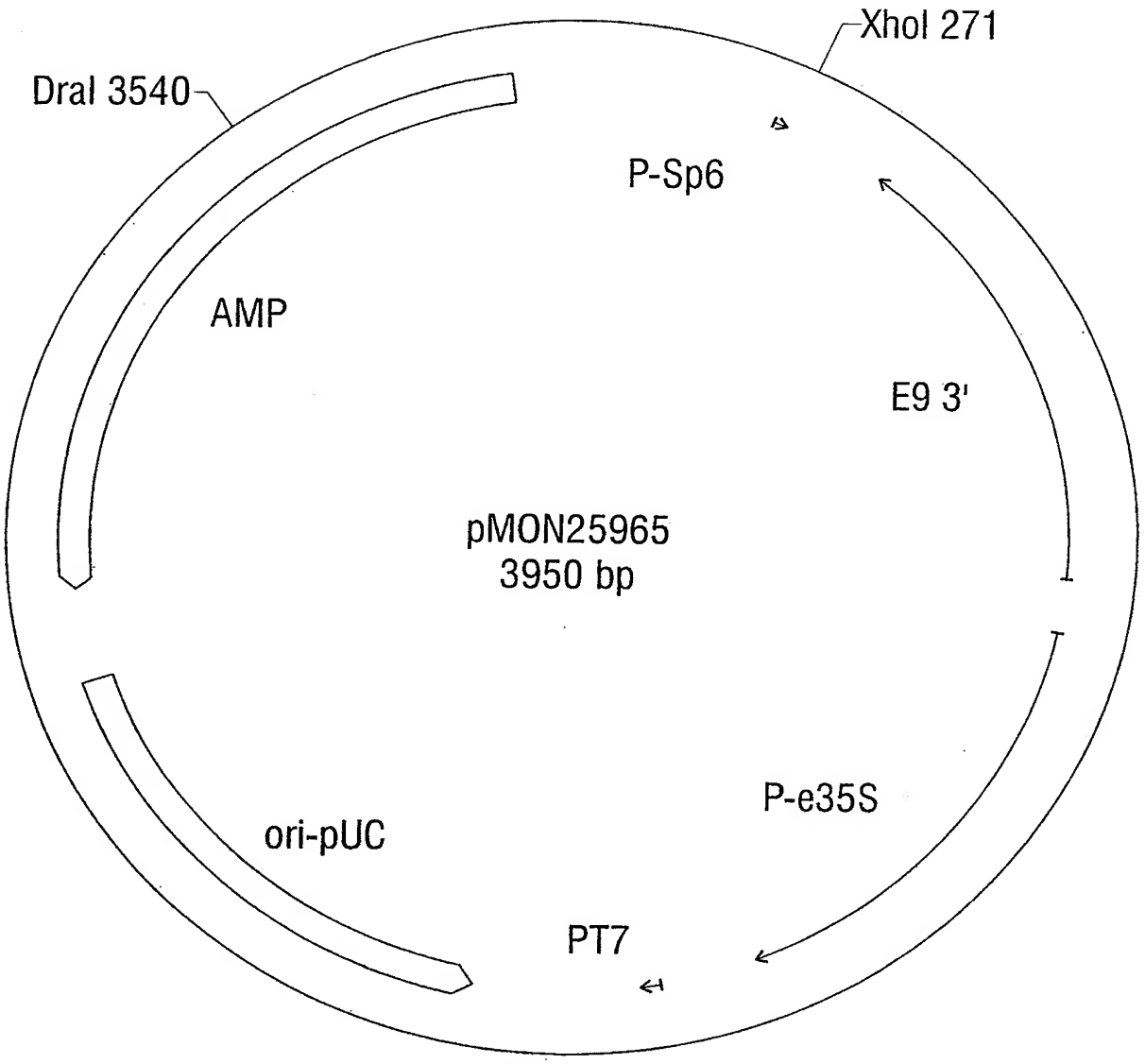
XhoI 271
DraI 3540
P-Sp6
AMP
E9 3'
pMON25965
3950 bp
P-e35S
ori-pUC
PT7

FIG. 30

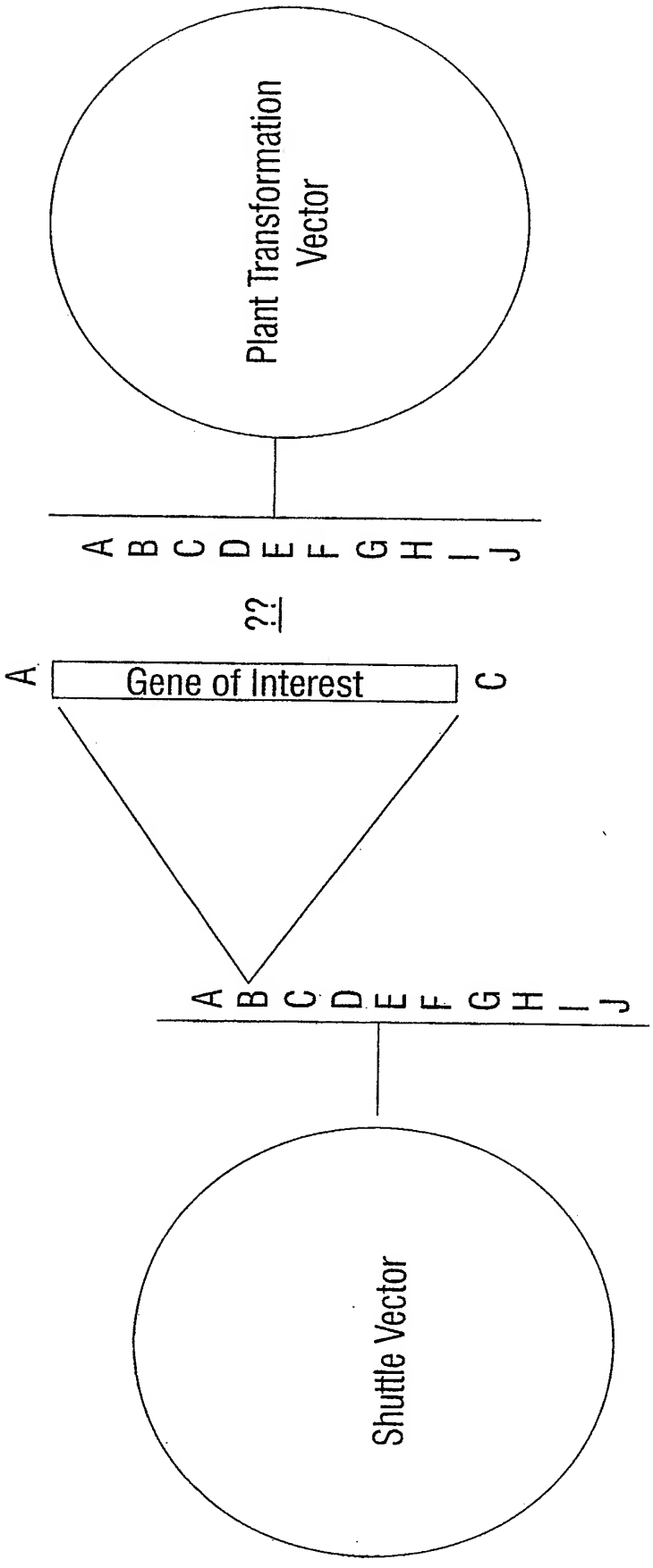
Plant Transformation Vector
A B C D E F G H I J
??
A
Gene of Interest
C
A B C D E F G H I J
Shuttle Vector

FIG. 31

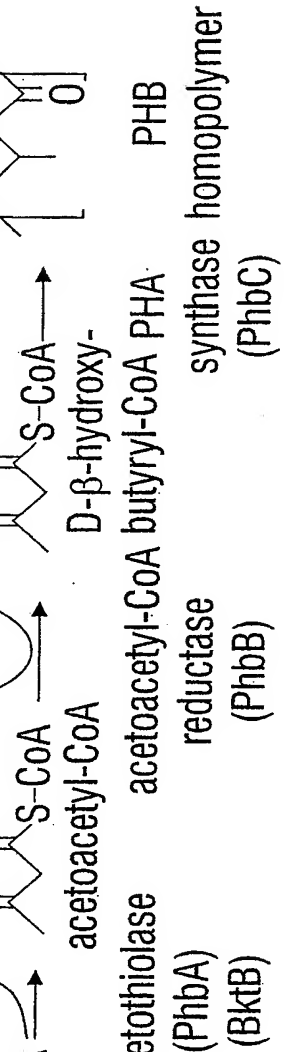
2 CH3–C–S–CoA
acetyl-CoA
CoA-SH
β-ketothiolase
(PhbA)
(BktB)
acetoacetyl-CoA
NADPH + H+ NADP+
acetoacetyl-CoA reductase
(PhbB)
D-β-hydroxy-butyryl-CoA
PHA synthase
(PhbC)
PHB homopolymer

FIG. 32

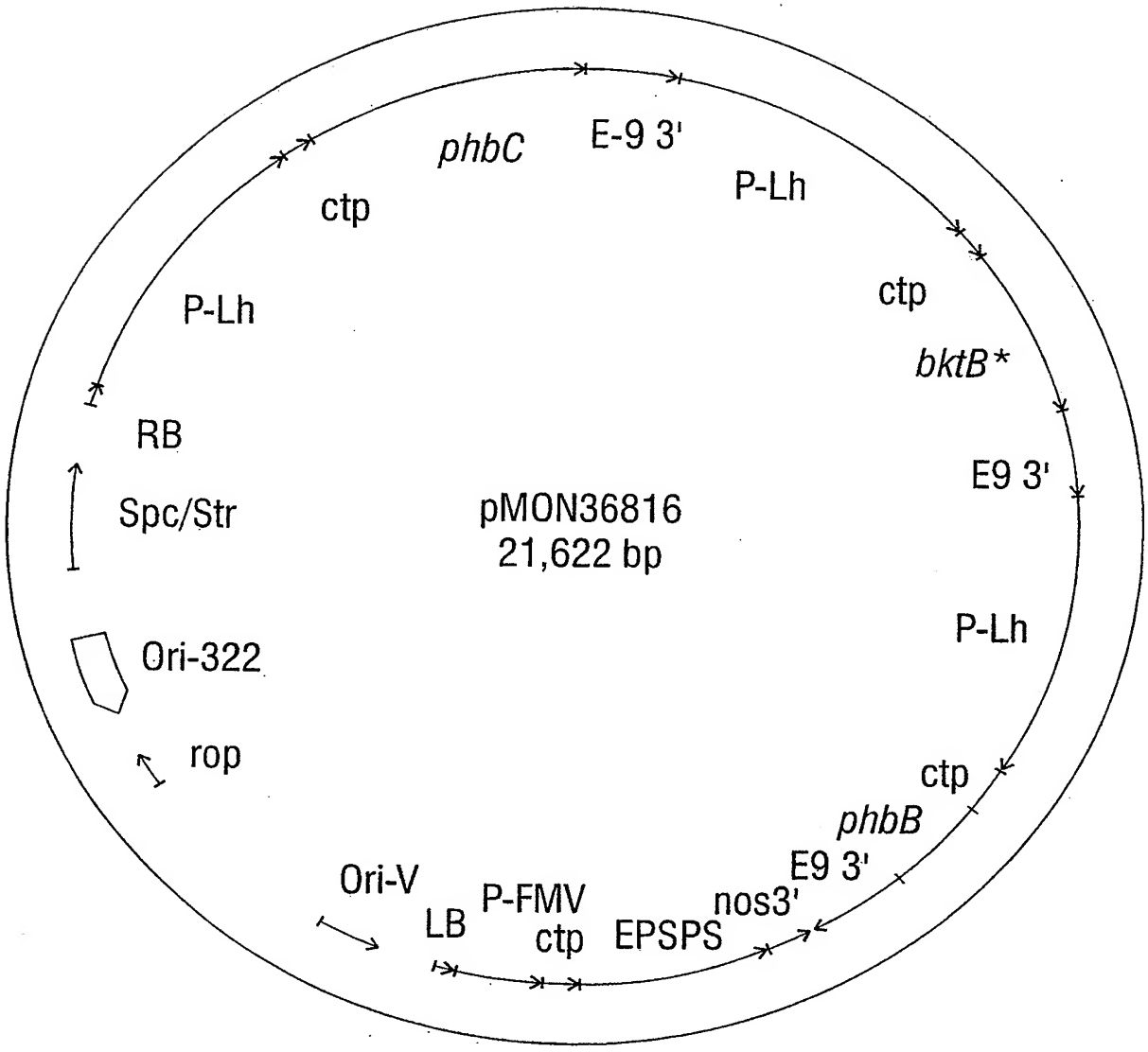
phbC
E-9 3'
P-Lh
ctp
P-Lh
ctp
bktB*
RB
E9 3'
Spc/Str
pMON36816
21,622 bp
P-Lh
Ori-322
rop
ctp
phbB
E9 3'
Ori-V
P-FMV
nos3'
LB
ctp
EPSPS

FIG. 33

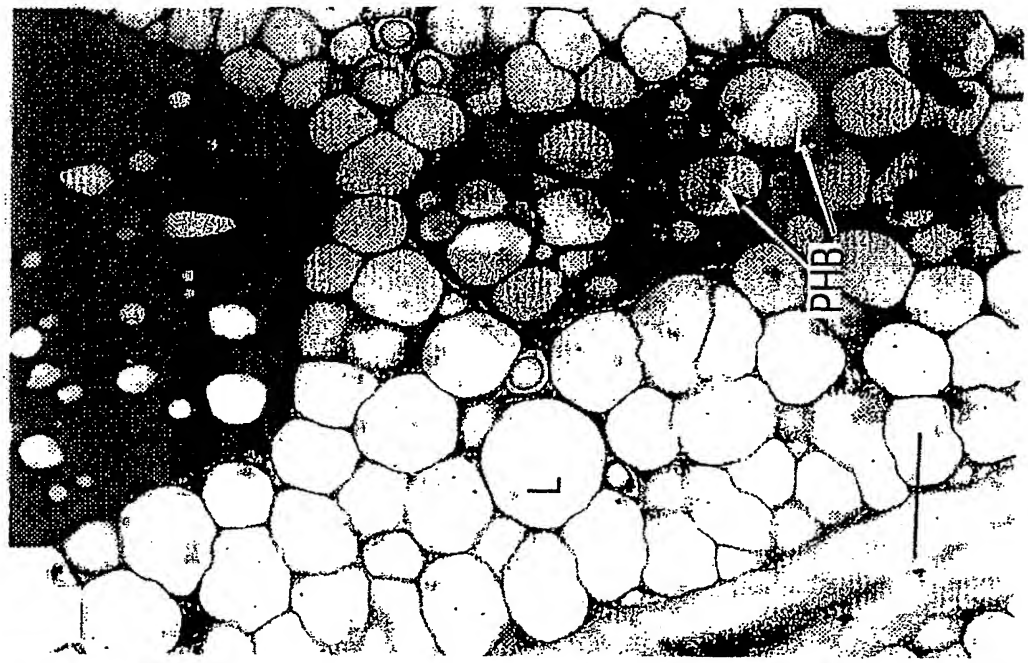

FIG. 34B

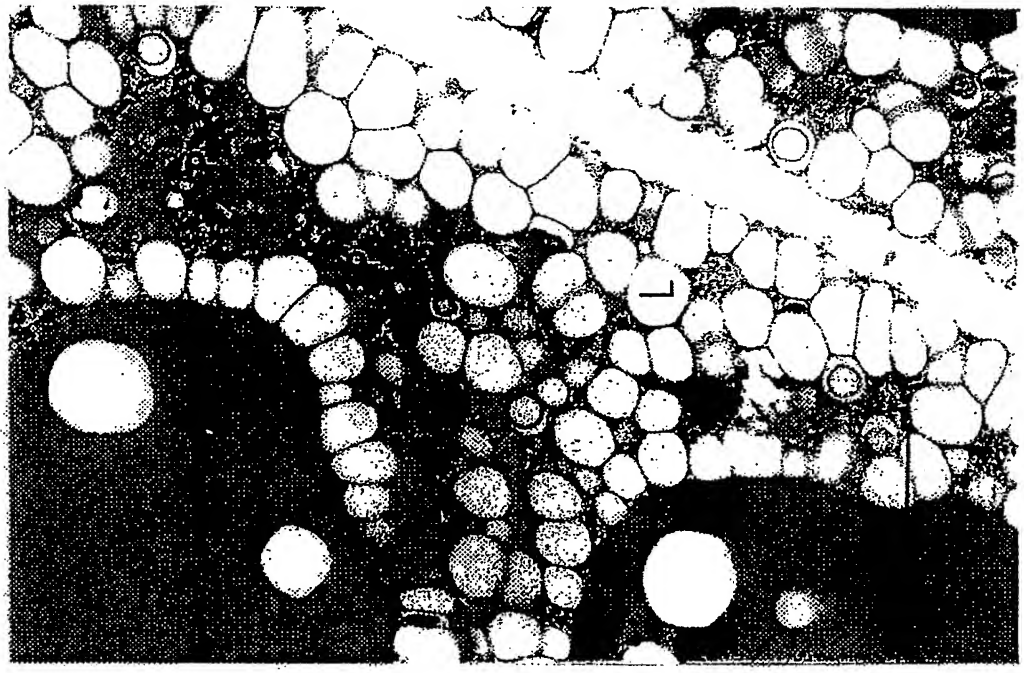

FIG. 34A

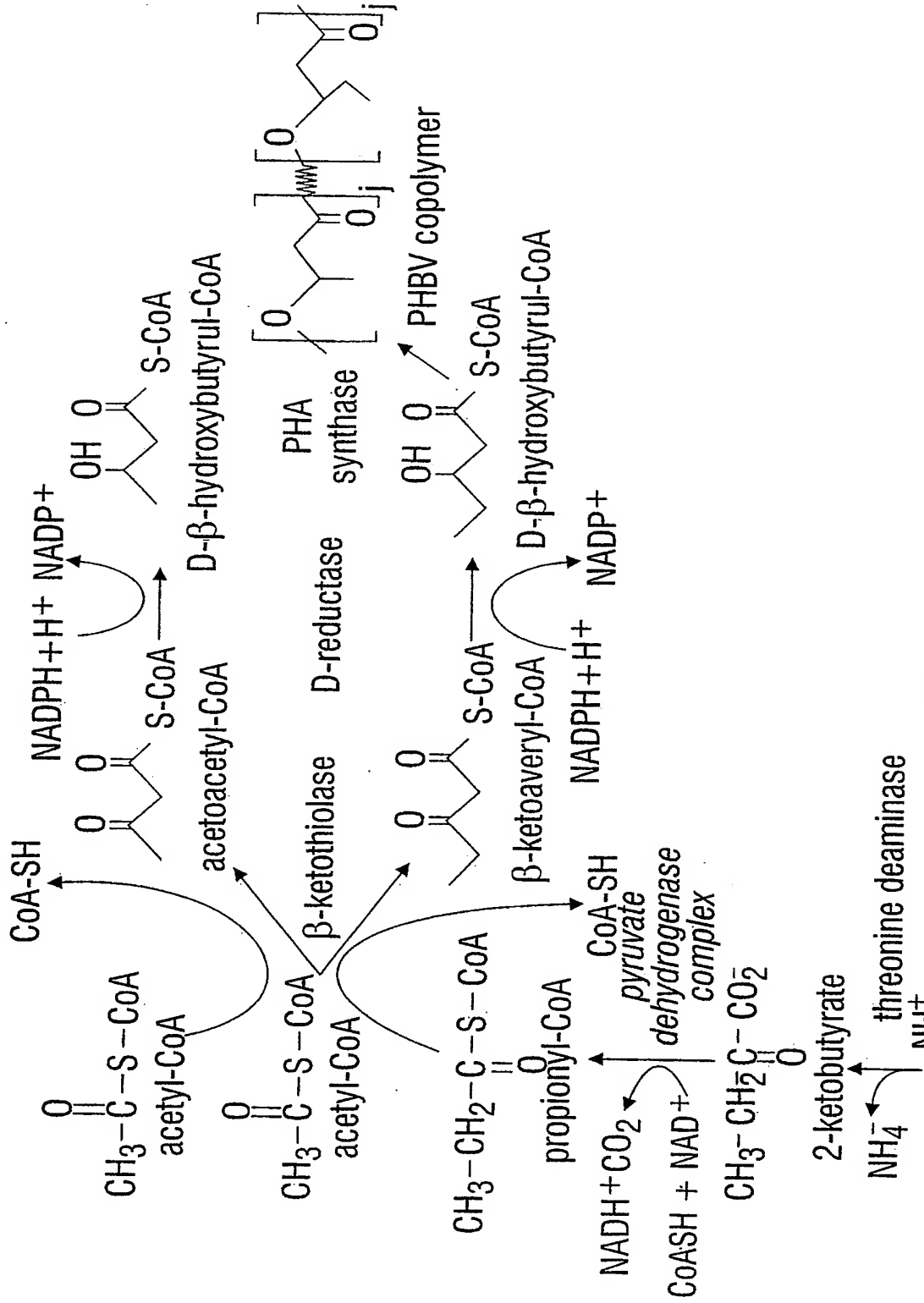
CoA-SH
acetyl-CoA
acetyl-CoA
β-ketothiolase
acetoacetyl-CoA
S-CoA
NADPH+H+ NADP+
D-β-hydroxybutyrul-CoA
D-reductase
PHA synthase
PHBV copolymer
β-ketoaveryl-CoA
S-CoA
NADPH+H+ NADP+
D-β-hydroxybutyrul-CoA
CoA-SH
pyruvate dehydrogenase complex
propionyl-CoA
NADH+CO2
CoASH + NAD+
2-ketobutyrate
threonine deaminase
NH4+
threonine

FIG. 35

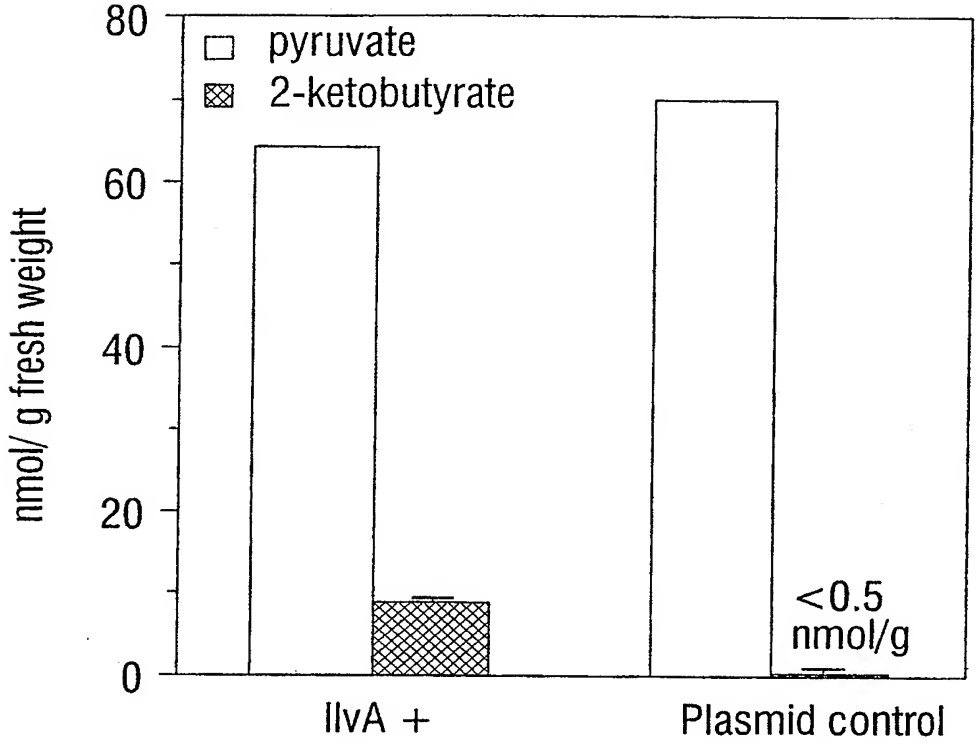

FIG. 36A

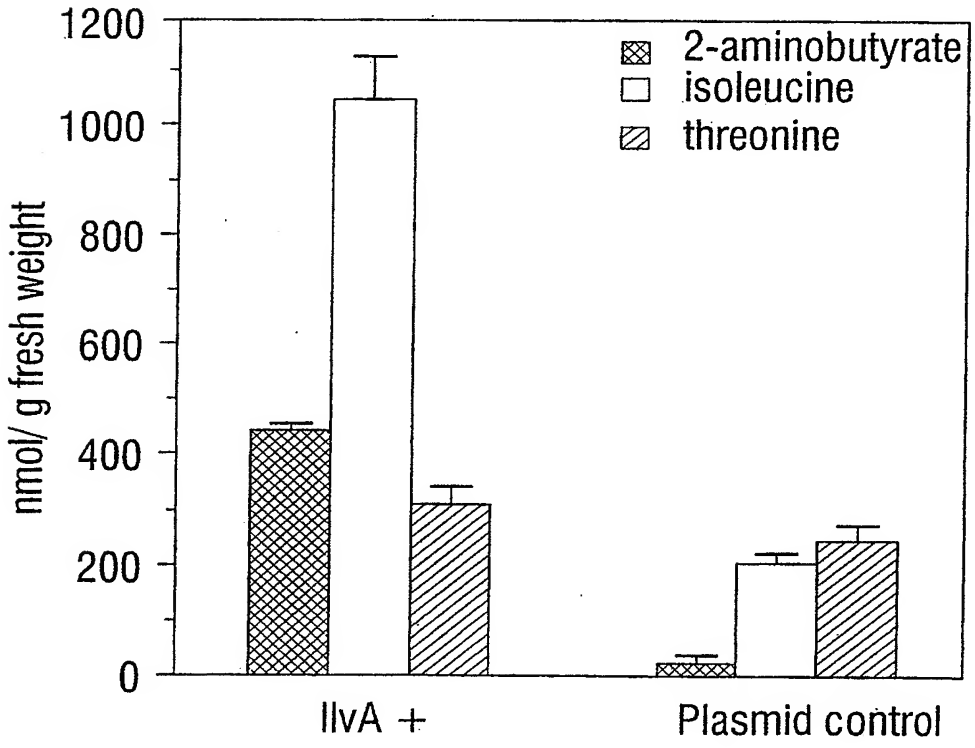

FIG. 36B

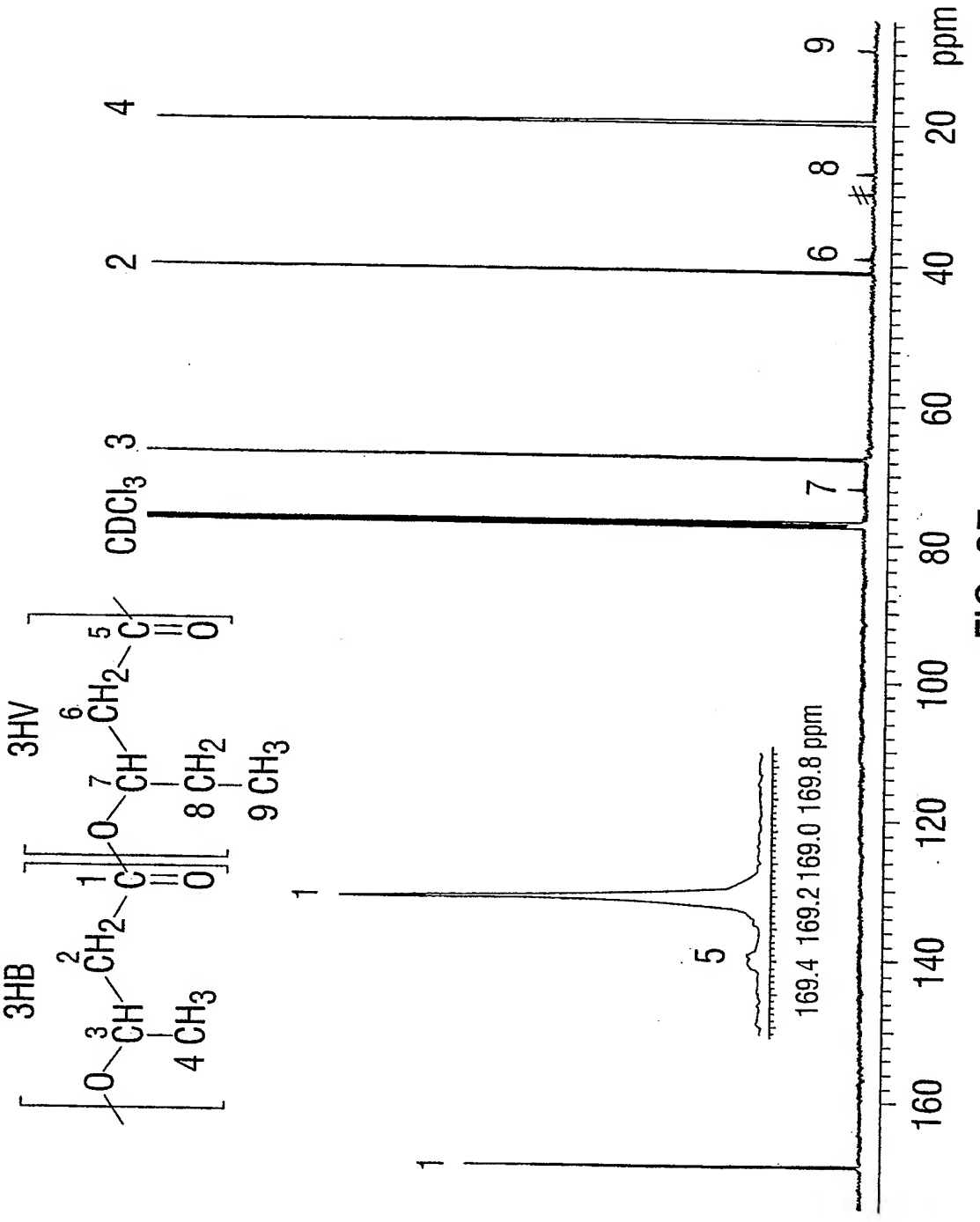

FIG. 37

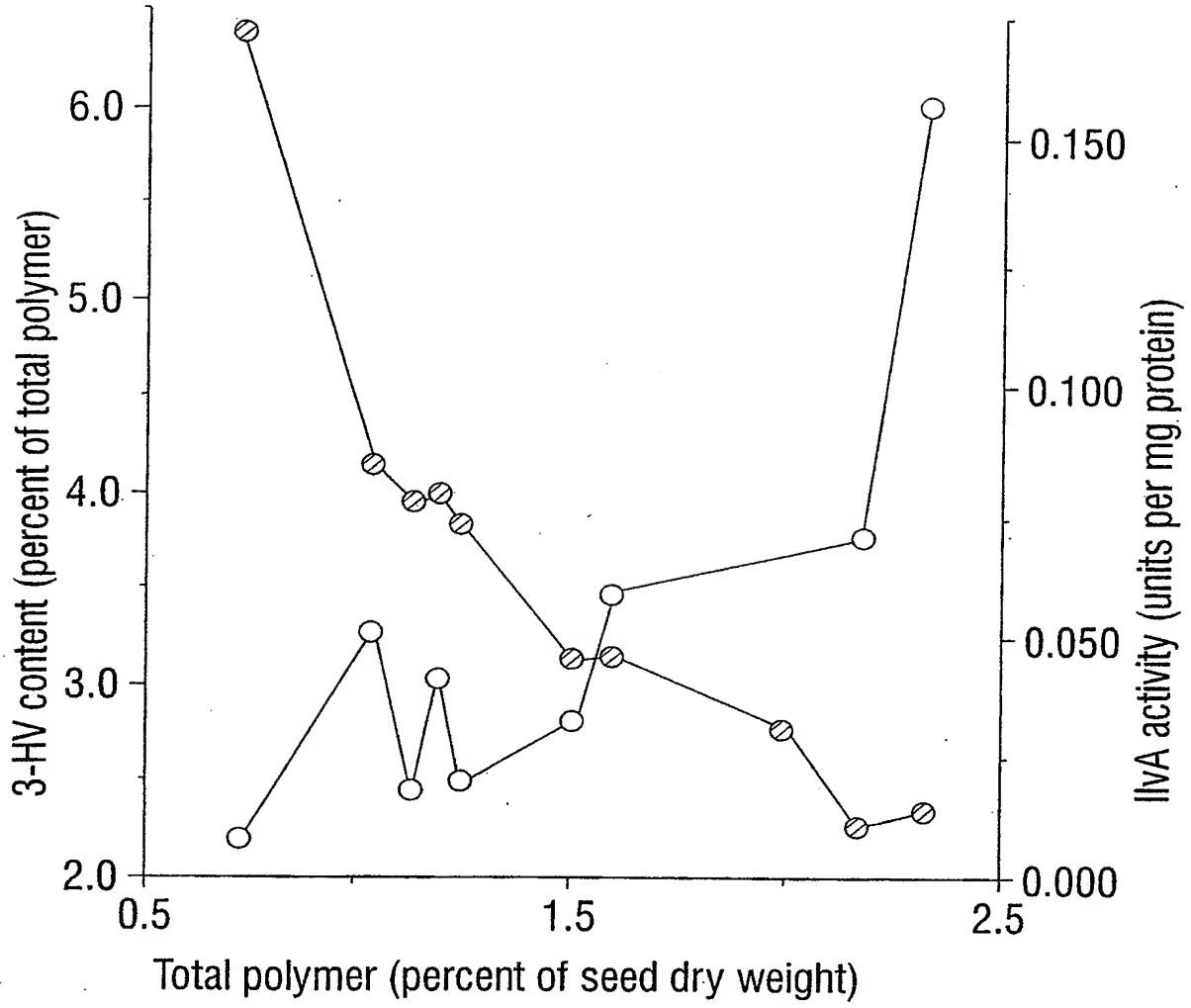
6.0
5.0
4.0
3.0
2.0
3-HV content (percent of total polymer)
0.150
0.100
0.050
0.000
IlvA activity (units per mg protein)
0.5
1.5
2.5
Total polymer (percent of seed dry weight)

FIG. 38

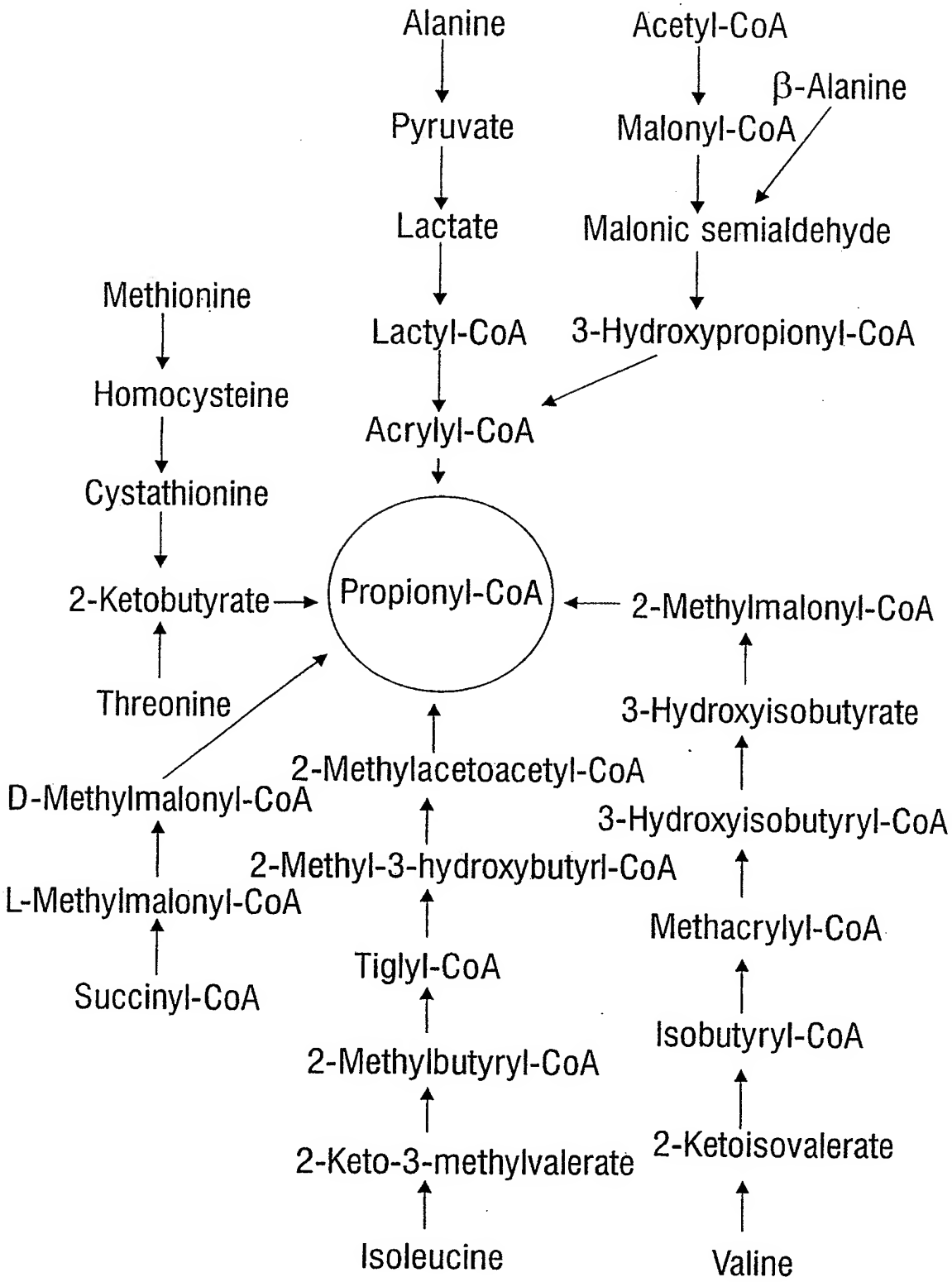
Alanine
Acetyl-CoA
β-Alanine
Pyruvate
Malonyl-CoA
Lactate
Malonic semialdehyde
Methionine
Lactyl-CoA
3-Hydroxypropionyl-CoA
Homocysteine
Acrylyl-CoA
Cystathionine
2-Ketobutyrate
Propionyl-CoA
2-Methylmalonyl-CoA
Threonine
3-Hydroxyisobutyrate
2-Methylacetoacetyl-CoA
D-Methylmalonyl-CoA
3-Hydroxyisobutyryl-CoA
2-Methyl-3-hydroxybutyrl-CoA
L-Methylmalonyl-CoA
Methacrylyl-CoA
Tiglyl-CoA
Succinyl-CoA
Isobutyryl-CoA
2-Methylbutyryl-CoA
2-Ketoisovalerate
2-Keto-3-methylvalerate
Isoleucine
Valine

FIG. 39

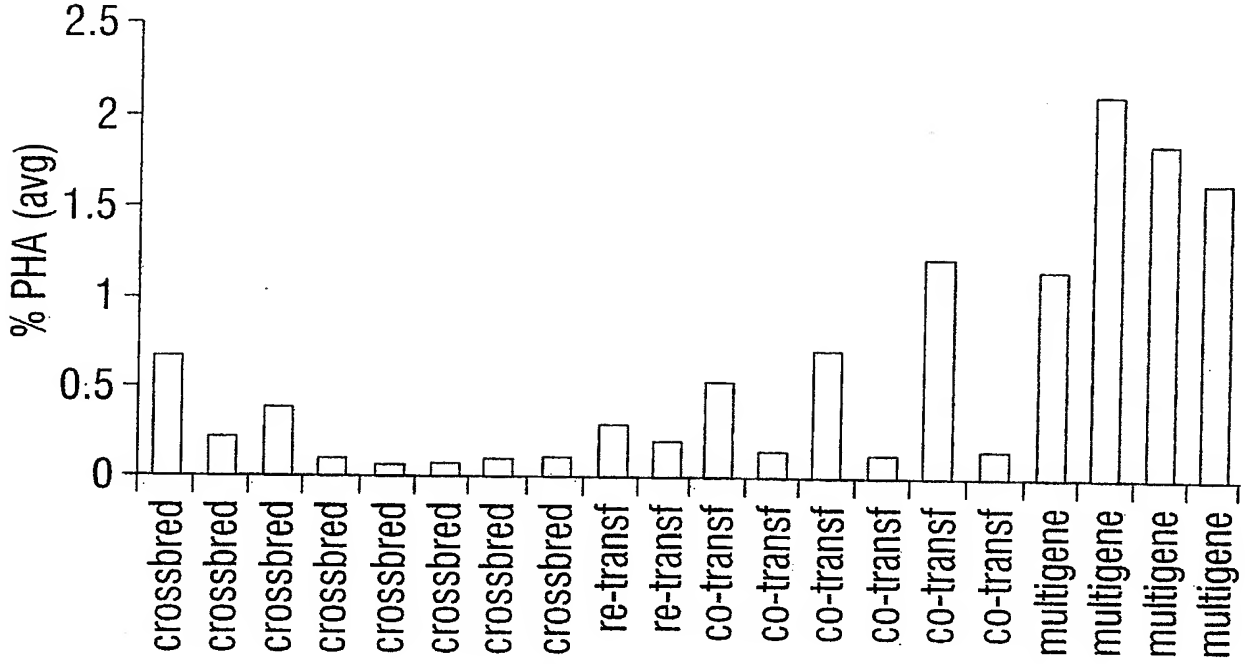
% PHA (avg)
2.5
2
1.5
1
0.5
0
crossbred
crossbred
crossbred
crossbred
crossbred
crossbred
crossbred
crossbred
re-transf
re-transf
co-transf
co-transf
co-transf
co-transf
co-transf
co-transf
multigene
multigene
multigene
multigene

FIG. 40

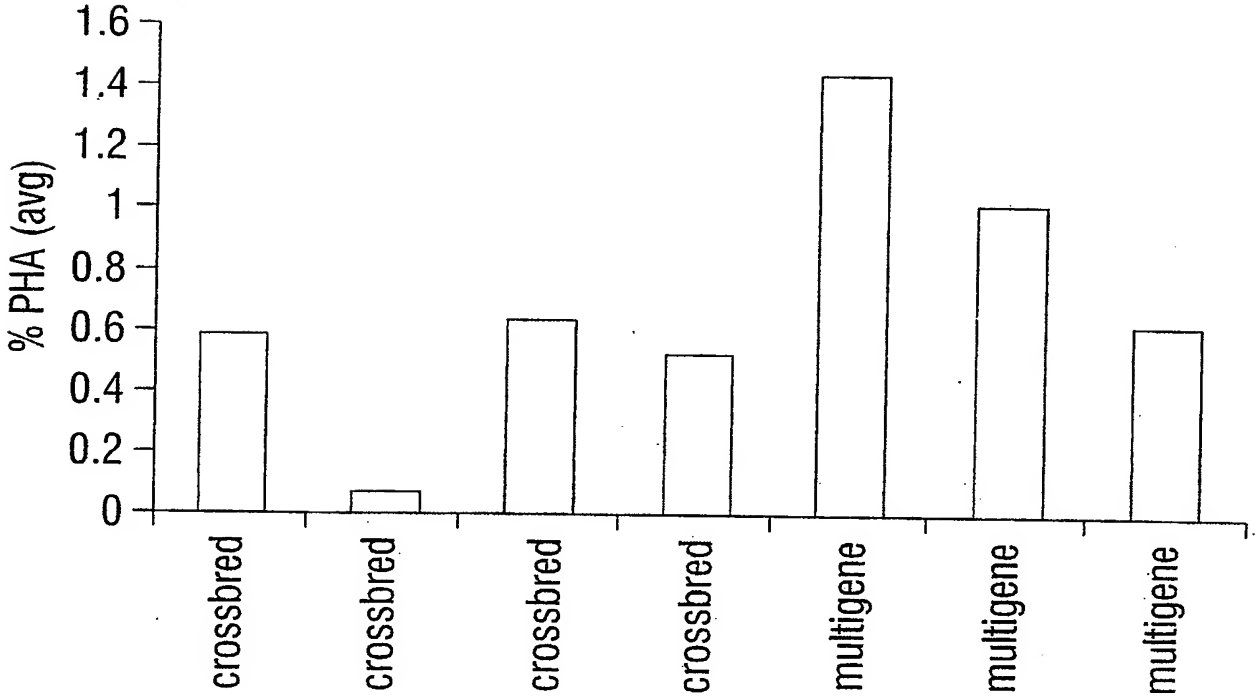
% PHA (avg)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
crossbred
crossbred
crossbred
crossbred
multigene
multigene
multigene

FIG. 41

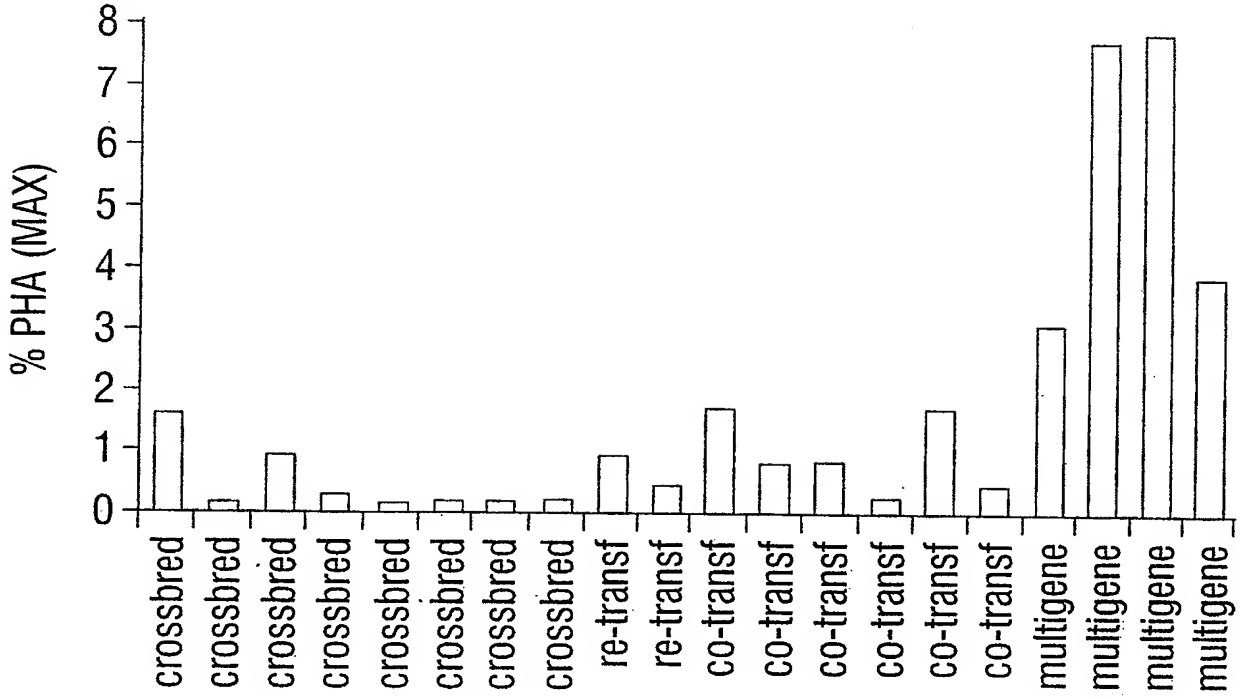
% PHA (MAX)
0
1
2
3
4
5
6
7
8
crossbred
crossbred
crossbred
crossbred
crossbred
crossbred
crossbred
crossbred
re-transf
re-transf
co-transf
co-transf
co-transf
co-transf
co-transf
co-transf
multigene
multigene
multigene
multigene

FIG. 42

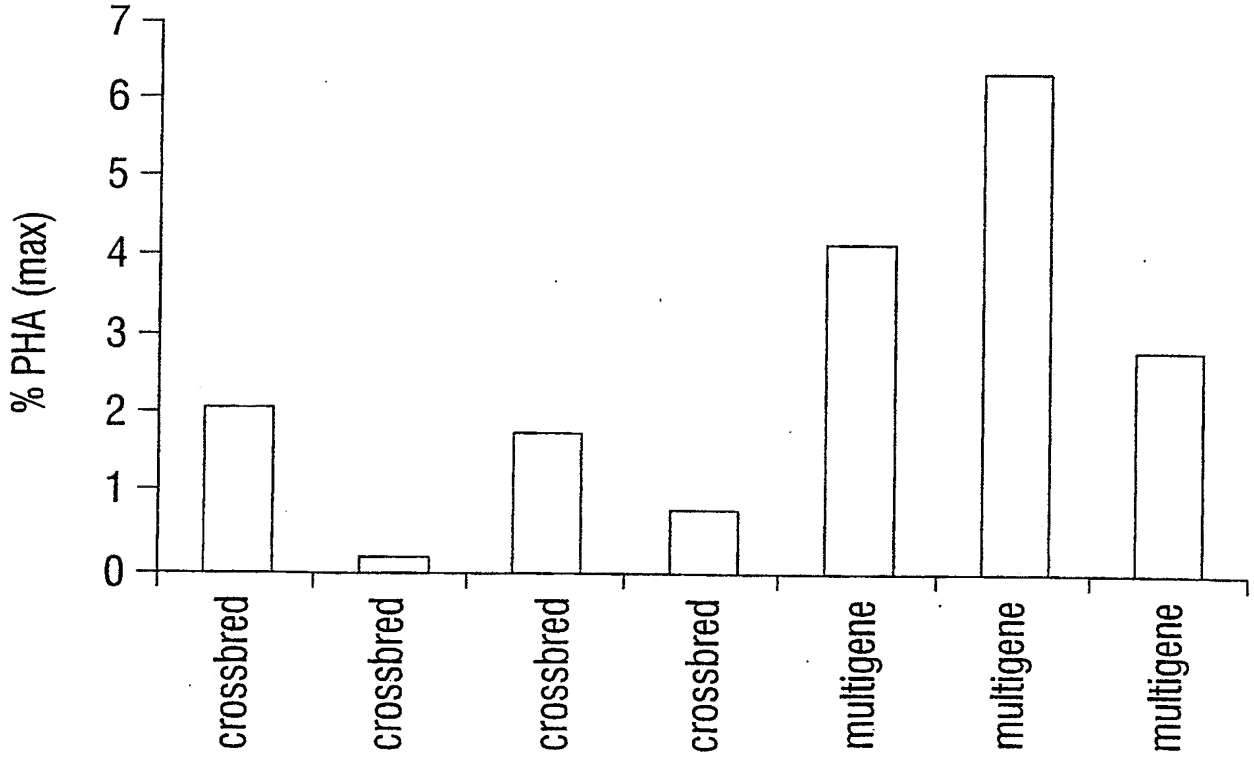
% PHA (max)
0
1
2
3
4
5
6
7
crossbred
crossbred
crossbred
crossbred
multigene
multigene
multigene

FIG. 43

INTERNATIONAL SEARCH REPORT

Inter ...nal Application No
PCT/US 00/05931

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 06225 A (INNOVATIVE TECH CENTER) 1 April 1993 (1993-04-01) page 14/ Fig. 5; page 15, page 18 ---	1,5,6,43
X	EP 0 870 837 A (MASSACHUSETTS INST TECHNOLOGY) 14 October 1998 (1998-10-14) page 13 ---	1,5,6,43
X	SCHUBERT P ET AL: "CLONING OF THE ALCALIGENES EUTROPHUS GENES FOR SYNTHESIS OF POLY-BETA-HYDROXYNUTYRIC ACID (PHB) AND SYNTHESIS OF PHB IN ESCHERICHIA COLI" JOURNAL OF BACTERIOLOGY,US,WASHINGTON, DC, vol. 170, no. 12, 1 December 1988 (1988-12-01), pages 5837-5847, XP002056793 ISSN: 0021-9193 abstract, page 5844, right column, Fig. 1 --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 July 2000	31/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Holtorf, S

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INTERNATIONAL SEARCH REPORT

Inter ɔnal Application No
PCT/US 00/05931

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 00557 A (MONSANTO CO) 8 January 1998 (1998-01-08) cited in the application see also pages 37 and 177 the whole document	1,2
A	WO 98 36078 A (UNIV MADISON ;DENNIS DOUGLAS E (US); VALENTIN HENRY E (US)) 20 August 1998 (1998-08-20) pages 3,5 , lines 13-18; page, page 29; 30,31, especially page 34, 35, line 1-2;	
A	WO 98 39453 A (SOEHLING BRIGITTE ;HEIN SILKE (DE); GOTTSCHALK GERHARD (DE); STEIN) 11 September 1998 (1998-09-11) page 32; example 5	
A	NAWRATH CHRISTIANE ET AL: "Targeting of the polyhydroxybutyrate biosynthetic pathway to the plastids of Arabidopsis thaliana results in high levels of polymer accumulation." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 91, no. 26, 1994, pages 12760-12764, XP002045228 1994 ISSN: 0027-8424 cited in the application the whole document	
A	WO 95 05472 A (UNIV MICHIGAN) 23 February 1995 (1995-02-23) the whole document	
A	NAWRATH C ET AL: "PLASTID TARGETING OF THE ENZYMES REQUIRED FOR THE PRODUCTION OF POLYHYDROXYBUTYRATE IN HIGHER PLANTS" STUDIES IN POLYMER SCIENCE,NL,ELSEVIER SCIENCE PUBLISHERS B.V., AMSTERDAM, vol. 12, 1994, pages 136-149, XP000564151 ISSN: 0922-5579 the whole document	
A	WO 92 19747 A (ICI PLC) 12 November 1992 (1992-11-12) cited in the application the whole document	

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1

INTERNATIONAL SEARCH REPORT

Inter ɔnal Application No
PCT/US 00/05931

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	BROUN ET AL: "Accumulation of ricinoleic, lesquerolic, and densipolic acids in seeds of transgenic Arabidopsis plants that express a fatty acyl hydroxylase cDNA from castor bean" PLANT PHYSIOLOGY,US,AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, ROCKVILLE, MD, vol. 113, no. 113, 1997, pages 933-942-942, XP002124104 ISSN: 0032-0889 cited in the application	
A	WO 98 33931 A (BERNDT CHRISTIANE ;BORMANN ERNST JOACHIM (DE); HILLIGER MATTHIAS () 6 August 1998 (1998-08-06) the whole document	
P,X	SLATER STEVEN ET AL: "Metabolic engineering of Arabidopsis and Brassica for poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer production." NATURE BIOTECHNOLOGY, vol. 17, no. 10, October 1999 (1999-10), pages 1011-1016, XP002142691 ISSN: 1087-0156 the whole document	1-4, 14-21, 25-30, 32-34, 36-41

1

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/US 00/05931

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		US 5569595 A	29-10-1996
EP 0870837 A	14-10-1998	AT 172497 T	15-11-1998
		CA 2062816 A	11-01-1991
		DE 69032713 D	26-11-1998
		DE 69032713 T	08-04-1999
		DE 870837 T	16-09-1999
		EP 0482077 A	29-04-1992
		ES 2125222 T	01-03-1999
		ES 2131489 T	01-08-1999
		JP 11332588 A	07-12-1999
		JP 5500751 T	18-02-1993
		WO 9100917 A	24-01-1991
		US 5480794 A	02-01-1996
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		AU 3144397 A	21-01-1998
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WO 9836078 A	20-08-1998	AU 6169498 A	08-09-1998
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		AU 7633094 A	14-03-1995
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		JP 9501832 T	25-02-1997
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		AU 1579792 A	21-12-1992
		CA 2109221 A	25-10-1992
		EP 0589898 A	06-04-1994
		JP 6510422 T	24-11-1994
		US 5502273 A	26-03-1996
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